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ISOLATION METHODS FOR MICROBIOLOGISTS

Edited by

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ISOLATION METHODS FOR MICROBIOLOGISTS

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Preface

THIS volume covers demonstrations included in the Autumn Demonstration Meeting of the Society for Applied Bacteriology held on 24th October 1967 at the Unilever Research Laboratories, Isleworth, Middlesex. It is No. 3 in the Technical Series and deals mainly with isolation techniques for aerobic micro-organisms. Selection of demonstrations was based on those microbial groups known to present special difficulties in isolation, and also on isolation techniques of more general interest and importance, and the demonstrators were asked to show the methods in use in their own laboratories.

No attempt is made to follow a pre-arranged comprehensive scheme since each contributor was invited to write a chapter based on his own techniques and demonstration, therefore each is a separate entity. In addition to the contributions of bacteriological interest, extra chapters are included dealing with the isolation of mycological micro-organisms which provide some methodological contrast.

Following the 1969 Autumn Demonstration Meeting it is considered that sufficient material should become available to compile Volume No. 4 in this Technical Series dealing mainly with methods for the isolation of anaerobic micro-organisms.

Our particular thanks are expressed to Mr. J. B. Wilkinson, Mr. R. C. S. Woodroffe and other members of the staff of the Unilever Research Laboratory, Isleworth, for their hospitality and help with the laboratory arrangements for the demonstrations. We would also like to thank Mr. A. Harry Walters for his help and advice.

January 1969

D. A. SHAPTON
G. W. GOULD

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The Use of Baird-Parker's Medium for the Isolation and Enumeration of *Staphylococcus aureus*

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During the last five years (1962–1967), more than twenty new or substantially modified media have been described for the isolation of *Staphylococcus aureus* from foods or clinical materials. Media for isolating *S. aureus* from foods have included those published by Baird-Parker (1962*a, b*), Crisley *et al.* (1964), Smuckler and Appleman (1964), Giolitti and Cantini (1966), Raj (1966), Sinell and Baumgart (1966), Madan (1967) and Rammell and Howick (1967). Media for isolating *S. aureus* from clinical materials include those described by Alder *et al.* (1962), Carantonis and Spink (1963), Grün and Müller (1964), Davis and Davis (1965), Blair *et al.* (1967) and Ruffo (1967). Recent reviews describing the relative performance of some of these media together with some of the more established media have been published by Neufeld and Garm (1963), McDivitt and Topp (1964), Marshall *et al.* (1965), Baer *et al.* (1966), Crisley *et al.* (1965) and Gilbert *et al.* (1969).

Baird-Parker's Medium

Baird-Parker's medium was devised for the specific detection and enumeration of *S. aureus* in foods. Since the publication of this medium (Baird-Parker, 1962*a*) it has been tested in laboratories in many countries and has been proposed as a standard medium for isolating *S. aureus* by at least one international commission, and several national public health authorities. Three commercial forms of the medium are currently available. Papers supporting the use of this medium have been published by Sharpe *et al.* (1962), Basille *et al.* (1964), Pablo *et al.* (1966), Thieulin *et al.* (1966), de Waart *et al.* (1968) and ten Broeke (1967). The medium has been shown to be particularly useful for recovering *S. aureus* from processed foods in which the organisms may have been damaged during drying, heating and/or irradiation and which are subsequently unable to grow on many of the media usually used for isolation; see Baird-Parker and Davenport (1965). In the following sections the preparation and use of the medium will be described in some detail.

Preparation of medium

The basal medium consists of the following by % w/v: Tryptone (Difco), 1.0; Lab-Lemco (Oxoid), 0.5; Yeast extract (Difco), 0.1; Lithium chloride (hydrated), 0.5; Agar (Difco), 2.0. The ingredients are dissolved in hot distilled or deionized water by steaming, and the pH adjusted to 6.8. The medium is then dispensed in 90 ml amounts, without filtration, into 4 oz screw-capped bottles and sterilized by autoclaving at 121° for 15 min; the pH after autoclaving should be 6.8–7.0.

The following four prewarmed and membrane-filter sterilized aqueous solutions are then added to 90 ml of the molten basal medium held at 45–50°: 6.3 ml of 20% w/v glycine; 1.0 ml of 1.0% w/v potassium tellurite (British Drug Houses; it is important to use this brand); 5.0 ml of 20% (w/v) sodium pyruvate; and 5.0 ml of Oxoid egg yolk emulsion. These solutions can be mixed together and added as a single mixture but the mixture should not be stored for any period. After the additions, 10 or 35 ml amounts of the medium are poured into 9 or 24 cm Petri dishes and allowed to set.

Storage of the medium

The basal medium can be stored indefinitely at room temperature in screw-capped bottles. Stock solutions of 20% glycine and 1% potassium tellurite can be stored for at least six months at room temperature but the 20% sodium pyruvate solution is best stored at 5° and should be renewed monthly.

Poured plates of the complete medium cannot be stored satisfactorily. Plates should therefore be freshly prepared and are best used within 24 h of pouring: they should be discarded if not used within 48 h. A stable version of the medium has been devised and is described later.

Use of the medium

Plates should be well-dried at 45–50° for 1 h before use. A 5–10% homogenate of the sample is prepared and a series of decimal dilutions made in 0.1 or 0.3% peptone water: 0.1 ml amounts of the homogenate and dilutions are pipetted to the centres of the plates and spread with sterile glass spatulas uniformly over the surfaces; spreading should be continued until the plate surfaces appear dry.

Interpretation and recording of results

Plates are incubated at 37° for up to 48 h. They should be stacked to ensure a good circulation of air around them otherwise the centre plates will take a



FIG. 1. Appearance of *Staphylococcus aureus* colonies on the medium.

long time to reach the incubator temperature. Plates should be first examined after a minimum incubation time of 24–26 h. Black and shiny colonies with narrow white margins surrounded by clear zones extending into the opaque medium (Fig. 1) are counted as specifically *S. aureus* and suitably marked on the plates. They are then incubated for a further 24 h and colonies with the appearance shown in Fig. 1 appearing on further incubation are tested for coagulase production as follows: the top part of the suspected colony is removed with a straight inoculating wire, suspended in 0.1 ml of heart infusion broth (Difco) in a $3 \times \frac{3}{8}$ in sterile test-tube, and incubated for 1 h at 37°. After incubation, 0.3 ml of Difco rabbit plasma is added and the tube reincubated for a further 2–6 h and then left overnight at room temperature; any degree of coagulation of the plasma is taken as evidence of coagulase

activity. The total *S. aureus* count is therefore the number of specific colonies on Baird-Parker's medium at 24 h plus those positive colonies confirmed by a coagulase test at 48 h. This method will detect 50 *S. aureus*/g of sample if 0.1 ml of a 10% homogenate is spread over the surface of each of two, 9 cm Petri dishes or 5/g if 1.0 ml amounts of the same macerate are spread over two 24 cm Petri dishes.

Enrichment

If it is desired to detect less than 5 *S. aureus*/g in a sample, an enrichment procedure should be used. Two procedures have been used with this medium, namely enrichment by incubation for 24–48 h in Robertson's meat broth + 10% NaCl (Maitland and Martyn, 1948; Baird-Parker, 1962b) or use of Giolitti and Cantini's (1966) medium (de Waart *et al.*, 1968).

Isolation from foods heavily contaminated with Proteus

The original medium does not suppress the growth and swarming of all *Proteus* spp. Therefore, for samples likely to be heavily contaminated with *Proteus* it is recommended that sulphamezathine should be added to the medium (Smith and Baird-Parker, 1964). The sulphamezathine can be added either to the base before autoclaving or as a membrane-filter sterilized solution that is added to the molten base together with the other additions, prior to the pouring of Petri dishes. In either case, an 0.2% w/v stock solution of the sodium salt of sulphamezathine is prepared by dissolving 0.5 g of pure sulphamezathine (Imperial Chemical Industries) in 25 ml of N/10 NaOH and making up to 250 ml with distilled water. This stock solution is used to give a final concentration in the medium of 50 µg/ml.

Growth of other organisms on the medium

Some Group D streptococci, micrococci, corynebacteria and members of Enterobacteriaceae grow on the medium and form black colonies (de Waart *et al.*, 1968); however, none of these organisms clear egg yolk. Other organisms such as yeasts, moulds and bacilli also grow but are readily recognized by the slate grey appearance of their colonies and their colonial morphology. Certain strains of *S. epidermidis* may also grow on the medium forming black colonies and cause clearing of the egg yolk. At 24 h colonies of such strains are usually distinguishable from *S. aureus* by a very wide opaque zone around them which in turn is surrounded by a very narrow clear zone. At 48 h, *S. aureus* may give a similar appearance and for this reason all late egg yolk clearers should be tested by the coagulase test.

Few bacteriologists appear to be aware that any citrate utilizing bac-

terium may cause non-specific clotting of the citrated plasmas commonly used for coagulase tests (Baird-Parker, 1965). Many members of the Enterobacteriaceae (Mushin and Kerr, 1954) and strains of faecal streptococci (Evans *et al.*, 1952) that utilize citrate will clot citrated plasma. Some of these organisms, particularly faecal streptococci, grow on Baird-Parker's medium but do not cause the characteristic clearing of the egg yolk. Therefore, if it is decided to test such colonies for coagulase production they should first be checked for catalase production (Baird-Parker, 1963). If negative, the colony can safely be assumed not to be *S. aureus*, as catalase negative strains of *S. aureus* are very uncommon. If positive, a smear stained by Gram's method should be examined microscopically.

Egg yolk reactions of Staphylococcus aureus

The mechanism of the egg yolk reaction has been studied in some detail by Lundbeck and Tirunarayanan (1966) and Tirunarayanan and Lundbeck (1967). They conclude that the egg yolk reaction observed on egg yolk agar arises from a sequence of events resulting from the action of a lipoprotein lipase on the lipovitellenin in the egg yolk. The first observable reaction is the clearing of the egg yolk (clear zone) which is then followed by the precipitation of calcium or magnesium salts of fatty acids in the area immediately surrounding the colony and the release of fatty substances at the agar surface. The diagnostic character of Baird-Parker's medium is based primarily on the clearing of egg yolk. The opaque zone, which is identical to the opacity produced by *S. aureus* growing in egg yolk broth (Gillespie and Alder, 1952) may be absent, particularly when colonies are examined after only 24 h incubation. About 90% of *S. aureus* strains isolated from food and all enterotoxin-producing strains so far tested produce the characteristic clearing of egg yolk after incubation on the medium for 24–26 h at 37°. A further 5–7% of strains cause clearing of egg yolk after incubation for a further 24 h. Although many of the antibiotic-resistant strains present in hospital environments are egg yolk negative (Willis *et al.*, 1966; Lowbury and Collins, 1964), there is ample evidence that such strains are uncommon in foods other than in certain dairy products. For example, de Waart *et al.* (1968) picked 165 black but egg yolk negative colonies off Baird-Parker's agar previously inoculated with a variety of foods, and then found only three of these were colonies of *S. aureus*.

Commercial Baird-Parker's Medium

The medium is currently manufactured by Baltimore Biological Laboratories (BBL), Difco Laboratories, and Oxoid Ltd. Oxoid Ltd., have pioneered the commercial form of the medium and it is extensively used in a

number of laboratories in this country and Europe. The more recent BBL medium appears to be very similar to that manufactured by Oxoid both in performance and composition. The Difco medium is very new but on the basis of a preliminary examination of two commercial samples it would appear to have several advantages over both the laboratory and the other commercial forms of the medium. These advantages are (1) only one addition is made to the medium prior to the pouring of the Petri dishes, (2) the medium is stable for at least 28 days at 5°, and (3) the medium appears to be more selective particularly with respect to *S. faecalis* and members of the Enterobacteriaceae. Further experiments have shown that certain batches of the medium are unstable; the manufacturers are investigating this problem.

Stable Laboratory Version of Baird-Parker's Medium

The laboratory version of the medium has two advantages over the commercial medium. First, it is considerably cheaper, and second, *S. aureus* generally grows better on the medium. We have been asked frequently if we could produce a stable form of the medium which can be stored as poured plates, since this would obviously be advantageous in a busy routine laboratory. We have recently achieved this by omitting the sodium pyruvate when the medium is poured into Petri dishes and adding it to the medium just prior to inoculation. 0.5 ml of the 20% w/v solution of sodium pyruvate is spread uniformly over the surface of a plate of Baird-Parker's medium (poured without pyruvate) and dried at 45–50° for 30 min–1 h. The Petri dish should be dried with the medium surface uppermost otherwise the pyruvate will run off. A commercial plate warmer is a cheap and convenient way for drying the poured Petri dishes. Poured Petri dishes without pyruvate have been stored for more than one month without sustaining any loss of selectivity or development of toxicity to *S. aureus* (Anderson *et al.*, 1969).

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Media for the Isolation and Enumeration of Coagulase-Positive Staphylococci from Foods

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The need to assess the bacteriological safety and quality of foods is now widely appreciated throughout the world. In 1962 the International Association of Microbiological Societies set up an International Committee on Microbiological Specifications for Foods (referred to in brief throughout this report as IAMS Committee), to investigate the possibility of introducing microbiological standards for certain foods. Because of rapidly expanding use of a diversity of media and methods for the detection and enumeration of many microbial species in foods the Committee have prepared a book of recommended techniques (IAMS Committee, 1968). The purpose of the present study was to compare the five selective media (Table 1, media 1-5) chosen by the IAMS Committee for the isolation and enumeration of coagulase-positive staphylococci from foods with three other selective media (Table 1, media 6-8) currently used in many laboratories. Table 1 gives the eight media and the countries in which they are mostly used. Blood agar was included as a non-selective control medium known to support good growth.

TABLE 1. Selective media for coagulase-positive staphylococci in foods

Number	Medium	Countries of origin and where used
1	Vogel and Johnson Agar	U.S.A.
2	Baird-Parker Medium	England, many countries
3	Egg Yolk-Sodium Azide Agar	Sweden
4	Tellurite-Polymyxin-Egg Yolk Agar	U.S.A.
5	Milk-Salt Agar (Russian formula)	U.S.S.R.
6	Milk-Salt Agar (English formula)	England
7	Staphylococcus 110 Medium	U.S.A., many countries
8	Phenolphthalein Diphosphate Agar with Polymyxin	England

Media

Vogel and Johnson Agar (VJA), Vogel and Johnson (1960). Base medium obtained from Baltimore Biological Laboratories Inc. and containing trypticase 1%, yeast extract 0.5%, mannitol 1%, dipotassium hydrogen phosphate 0.5%, lithium chloride 0.5%, glycine 1%, agar 1.6%, phenol red 0.0025%. For use add potassium tellurite (B.D.H.) 1% solution, 2%.

Baird-Parker Medium (BPM), Baird-Parker (1962). Base medium obtained from Oxoid Ltd. and containing tryptone 1%, Lab-Lemco beef extract 0.5%, yeast extract 0.1%, sodium pyruvate 1%, glycine 1.2%, lithium chloride 0.5%, agar 2%. For use add concentrated egg yolk emulsion (Oxoid) 5% and potassium tellurite (B.D.H.) 3.5% solution, 0.3%.

Egg Yolk-Sodium Azide Agar (EYAA), Lundbeck and Tirunarayanan (1966), modified formulae (IAMS Committee, 1968). Base medium contains peptone 1%, Lab-Lemco beef extract 0.55%, sodium chloride 0.3%, disodium monohydrogen phosphate dodecahydrate 0.02%, agar 1.5%. For use add sodium azide 0.015% and egg yolk: saline emulsion (1:1), 15%.

Tellurite-Polymyxin-Egg Yolk Agar (TPEY), Crisley, Angelotti and Foter (1964). Base medium contains tryptone (Difco) 1.1%, yeast extract 0.55%, mannitol 0.55%, sodium chloride 2.2%, lithium chloride 0.22%, agar 2%. For use add to 90 ml of base, Polymyxin B sulphate (Burroughs Wellcome) 1% solution 0.04 ml, egg yolk: saline emulsion (3:7) 10 ml and potassium tellurite (B.D.H.) 1% solution, 1 ml.

Milk Salt Agar, Russian formula (MSAR), Laboratory Diagnosis of Infectious Diseases; methodological manual, Moscow (1964). Base medium contains peptone 0.5%, Lab-Lemco beef extract 0.3%, sodium chloride 6.5%, agar 1.5%. For use add sterile skim milk 10%.

Milk Salt Agar, English formula (MSAE), modification of Crones medium (pers. commun.). Base medium contains peptone 0.5%, yeastrel 0.3%, sodium chloride 6.5%, agar 1.2%. For use add pasteurized homogenized milk 10%.

Staphylococcus Medium 110 (110M), Chapman (1946). Medium obtained from Oxoid Ltd. and containing tryptone 1%, yeast extract 0.25%, lactose 0.2%, mannitol 1%, sodium chloride 7.5%, dipotassium hydrogen phosphate 0.5%, gelatin 3%, agar 1.5%.

Phenolphthalein Phosphate Agar with Polymyxin (PPAP), modified after Barber and Kuper (1951) by Smith (pers. commun.). Base medium contains peptone 1%, Lab-Lemco beef extract 1%, sodium chloride 0.5%, agar 1%. For use add phenolphthalein diphosphate pentasodium salt (Koch-Light) 0.5% solution, 2% and Polymyxin B sulphate (Burroughs Wellcome) 125 I.U. per ml.

TABLE 2. Comparison of eight selective media for the enumeration of coagulase-positive staphylococci as a percentage of counts on BA at 35°

Staphylococcus culture	Source of culture	Plate count × 10 ⁶ /ml	Counts (24 and 48 h) from 24 h nutrient broth on selective media expressed as a percentage of counts on BA																
			BA 24h 48h		VJA 24 48		BPM 24 48		EYAA 24 48		TPEY 24 48		MSAR 24 48		MSAE 24 48		11OM 24 48		PPAP 24 48
CIRL _J /62/10312	Almond paste	200 200		212 187	125 125	112 112	125 125	112 112	125 125	112 112	150 150	200 200	87 112						
	cake																		
CIRL _J /62/10426	Cooked Ham	500 525		110 110	105 100	90 86	105 100	110 105	70 67	100 110	65 62								
CIRL _J /62/10701	Chicken	525 550		114 114	81 77	119 109	57 55	148 136	62 59	95 86	105 109								
CIRL _J /62/12146	Fish Fingers	175 175		186 186	100 100	129 129	214 171	86 86	143 143	100 100	114 114								
CIRL _J /62/12148	Corned Beef	375 375		93 93	113 100	100 100	113 113	93 93	40 47	53 53	53 53								
CIRL _J /63/16802	Faeces	425 425		53 53	88 88	76 76	59 59	118 59	76 76	41 41	82 82								
FH/67/8006	Cooked Ham	200 200		32 32	77 75	50 50	87 87	112 112	42 42	69 67	75 87								

TABLE 3. Effect of experimental contamination on recovery of coagulase-positive staphylococci from fresh cream

Staphylococcus culture	Average counts $\times 10^2$ (24 and 48 h) of staphylococci from fresh cream inoculated with staphylococci and contaminants									
	Total plate count on BA		Selective media							
	24	48	VJA	BPM	EYAA	TPEY	MSAR	MSAE	IIOM	PPAP
	24	48	24	48	24	48	24	48	24	48
CIRL/62/10312	275	300	40	35	17	ND	TS	TS	35	35
CIRL/62/10426	350	350	50	60	ND	ND	TS	TS	TS	47
CIRL/62/10701	325	350	67	70	62	ND	TS	TS	87	75
CIRL/62/12146	200	200	57	65	PD	ND	TS	TS	TS	42
CIRL/62/12148	525	550	40	45	PD	ND	TS	TS	TS	37
CIRL/63/16802	525	525	TS	55	PD	ND	TS	TS	47	72
FH/67/8006	150	150	20	22	12	ND	TS	TS	17	22

TS Too small to count

PD Poor differentiation, plate count unreliable

ND No differentiation, plate count unreadable

Blood Agar (BA) Nutrient broth No. 2 (Oxoid), agar 1.2%, defibrinated horse blood 5%. This is poured onto a thin layer of peptone water agar.

Cultures

Sources of the six food poisoning strains of coagulase-positive staphylococci obtained from the Cross-Infection Reference Laboratory, Colindale (CIRL), and one coagulase-positive staphylococcus from the Food Hygiene Laboratory (FH) are listed in Table 2.

Recovery Studies

A modified Miles and Misra counting method with quarter-strength Ringer's solution as diluent was used throughout. Duplicate plates of each medium were used, one being incubated at 35° for 24 h and the other at 35° for 48 h.

In the studies of recovery, of staphylococci from experimentally contaminated foods, approximately equal numbers of each of three or four common bacterial food contaminants were added with the coagulase-positive staphylococci to each of two non-sterile foods (cooked ham and fresh cream). The contaminants were micrococci, coliform bacilli and aerobic sporing bacilli (all food isolates) and a coagulase-negative staphylococcus NCTC 7292.

Results and Discussion

Isolation of pure cultures of coagulase-positive staphylococci

Table 2 shows the counts from 24 h nutrient broth cultures of the seven strains of staphylococci on the eight selective media. The results are expressed as percentage values of the counts observed on Blood Agar. Results from 5 h nutrient broth cultures gave essentially similar results expressed as percentages but the level of counts was much lower.

Recovery of coagulase-positive staphylococci from experimentally contaminated foods

Table 3 shows the colony counts of coagulase-positive staphylococci from the artificially contaminated non-sterile cream enumerated on the selective media. Experiments with artificially contaminated cooked ham gave similar results.

Colonies on MSAR and MSAE were too small to count after 24 h incubation and a longer incubation time was required. To a lesser extent the same was true for VJA and 110M. MSAR and MSAE rely partially on

pigmentation of coagulase-positive staphylococci for differentiation and this character varied considerably from strain to strain.

Selectivity of coagulase-positive staphylococci was especially weak on EYAA, because the inhibition of contaminants was poor or lacking. VJA, BPM, TPEY and PPAP suppressed the mixed flora of contaminating bacteria better than MSAR, MSAE and 110M. BPM was the most able to suppress coagulase-negative staphylococci. A highly selective medium is normally required to suppress the heterologous microflora associated with many foods.

Although the reconstituted base of BPM can be stored satisfactorily for long periods the plates must be used within 24 h of being poured (Baird-Parker, 1962). Plates of all the other media could be stored satisfactorily with no apparent loss of functions for at least five days at 4°. In particular plates of PPAP can be stored satisfactorily for 14 days or more at 4° (Hobbs *et al.*, 1968).

The preparation of MSAR, MSAE, PPAP and two of the dehydrated media VJA and 110M was simple and involved less time than that required for BPM and TPEY.

Comparative costing of the media show that VJA, BPM, TPEY and EYAA were more expensive per plate than PPAP, 110M, MSAR and MSAE. The milk-salt media were particularly cheap and easy to prepare.

The present study was limited but intended to complement other more extensive evaluations (Baird-Parker, 1962; Crisley *et al.*, 1965; Baer *et al.*, 1966). For normal routine work a reliable, selective medium is required which is cheap, and simple to prepare and stable on storage. For several years we have used PPAP; MSAR appears to be a useful medium too. For research and development studies a more sophisticated medium may be required; BPM or TPEY are probably the most suitable.

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Bismuth Sulphite Media in the Isolation of Salmonellae

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Direct plating on a bismuth-sulphite medium on which salmonellae produce a characteristic colony makes possible the isolation and identification of salmonellae within 24 h of the receipt of a sample from an acute infection. For other samples (human and animal foods; crude sewage, sewage effluent, sewage-polluted natural waters), in which salmonellae are present in much smaller numbers than in acute infections, enrichment of the sample is usually necessary. With enrichment the earliest time at which a positive result is obtained is 48 h from receipt of sample. A negative report cannot confidently be made in less than 72 h from receipt of sample.

The Characteristic Salmonella Colony

On suitable bismuth-sulphite agar, well separated salmonellae produce after 18 h incubation colonies characterized by a jet-black circular centre with sharp edge surrounded by a clear translucent periphery. On cross-section the colonies are usually convex; but flat, umbilicate, or circumvallate colonies are not unusual. All these varieties may be encountered in a pure culture of a single serotype. Rough colonies are rare. The ratio of black centre to clear translucent edge varies according to the serotype. *Salmonella oranienburg* and most strains of *S. paratyphi B*, for example, form large mucoid colonies in which the clear translucent edge is minimal.

On plates from materials heavily contaminated with other *Enterobacteriaceae*, particularly *Proteus sp.*, the clear translucent edge of salmonella colonies may appear brownish due to diffusion in the medium of growth products of the other organisms. The jet-black centre with sharp edge of the salmonella colony, however, remains unaltered.

The salmonella colony may be surrounded by a mirror of precipitated bismuth, which is, however, common to all organisms which produce hydrogen sulphide and is not therefore confined to salmonellae. The mirror is useful in directing attention to colonies. This mirror is not a prominent

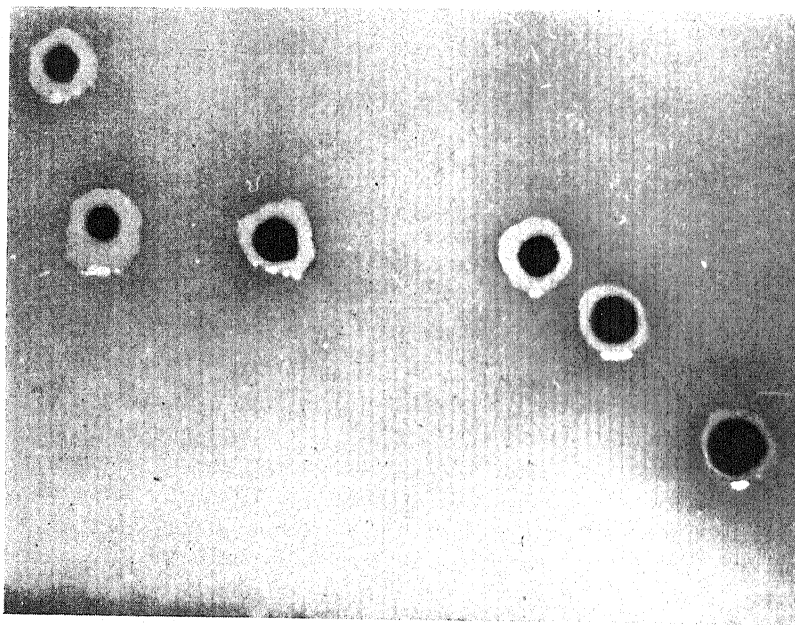


FIG. 1. The characteristic salmonella colony $\times 8$, on aged bismuth sulphite agar after 18 h incubation. "Mirrors" around some of the colonies are shown as a darkening of the medium.

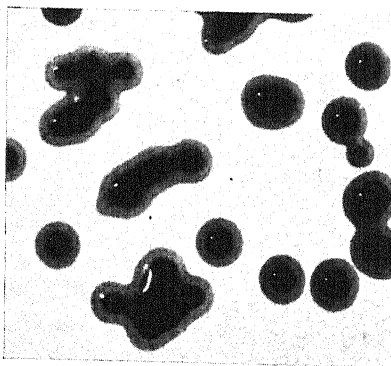


FIG. 2. The mucoid type of characteristic salmonella colony $\times 8$ on aged bismuth sulphite agar after 18 h incubation. "Mirrors" are absent.

feature in media using iron citrate. In salmonella colonies the jet-black colour of the centre of the colony extends to the medium underneath. The "mirror" surrounding the colony does not extend beneath the translucent edge of the colony. On wiping off a characteristic colony a "mirror image" of the colony remains on the surface of the medium. Figures 1 and 2 show characteristic colonies on bismuth-sulphite agar.

Only four serotypes, *S. gallinarum*, *S. pullorum*, *S. cholerae-suis*, and *S. paratyphi A*, fail to produce characteristic colonies. These serotypes on bismuth-sulphite media produce clear translucent colonies which may or may not be coloured green by the absorption of dye from the medium. The dye absorbed may be concentrated in the centre of the colony or may be evenly distributed throughout. An occasional strain of *S. typhi-murium* or *S. paratyphi B* may fail to produce characteristic colonies.

For the development of the characteristic colony good separation of colonies is necessary. In practice, inoculation of a whole plate with a wet loop has been found satisfactory. The use of a hand lens for examining plates is essential. A single salmonella colony on a plate heavily crowded with other organisms can easily be identified.

The Medium

Wilson (1923) showed that in the presence of a fermentable carbohydrate salmonellae in pure culture reduced sodium sulphite with the production of hydrogen sulphide. Using ferric chloride as indicator, colonies of organisms reducing sulphite appeared black. The reaction occurred within a few hours of inoculation and proved a useful confirmatory medium but could not be used for the direct isolation of salmonellae as other intestinal organisms also produced dark colonies.

Wilson and Blair (1927) found that a combination of bismuth and sodium sulphite afforded an enrichment and selective medium for *S. typhi* which at the same time partially or completely suppressed the growth of *Escherichia coli*. In this medium ferrous sulphate was used as indicator of the production of hydrogen sulphide. The bismuth salt used was bismuth citrate dissolved in ammonia.

In 1938, Wilson reported the isolation of *S. typhi* in water and milk-borne epidemics by means of bismuth-sulphite media and recommended several changes in the medium, notably the use of bismuth-ammonia-citrate scales and the substitution of iron citrate for ferrous sulphate. For the isolation of surface colonies, inoculation of the medium immediately on setting after pouring was recommended. Isolated colonies of *S. typhi* and *S. paratyphi B* were described as black: typhoid colonies appearing usually within 24 h, paratyphoid B colonies within 48 h.

The necessity to incubate plates of this medium for 48 h for the appearance of salmonella colonies other than *S. typhi* arises directly from the recommendation to inoculate plates immediately after the medium had set. Cook (1952) showed that freshly poured plates of bismuth-sulphite agar were inhibitory, in some cases supporting the growth of only one organism per million of those growing on blood agar. The inhibition decreased as the medium aged and small numbers of organisms usually grew well on plates that had been stored in the refrigerator for four or five days. Ageing of bismuth-sulphite agar was found to be accompanied by a gradual deepening of the green colour of the medium indicating that the brilliant green, reduced to its leuco-compound by the sulphite, was being oxidized. The thinner the layer of medium on the plates, the more rapidly the green colour returned.

The medium used is aged in the refrigerator for 3-4 days after pouring. In addition to the colour change described above, the most useful aid to the recognition of properly aged medium is the development of a characteristic sweet odour resembling that of violets. Freshly poured inhibitory media have a sour acid smell.

Use of the Medium

Direct inoculation

Samples of excreta from man and animals are examined both by direct plating and by enrichment before plating.

In acute infections, the normal flora of the gut is replaced almost completely by salmonellae. Direct plating of such samples seldom fails to reveal salmonellae. In convalescence the numbers of salmonellae fall and finally disappear as the normal flora is re-established. Direct examination alone may therefore fail to detect the convalescent or chronic carrier excreting minimal numbers of salmonellae.

Enrichment is required for materials containing small numbers of salmonellae in association with large numbers of other intestinal micro-organisms.

The enrichment media recommended are Selenite F medium (Hobbs and Allison, 1945; Smith, 1959), Rolfe's (1946) B modification of tetrathionate broth, and the magnesium chloride broth of Rappaport *et al.* (1956). Selenite enrichment medium is essential for the isolation of *S. typhi*. Tetrathionate can be modified to support growth of *S. typhi* but this renders it less satisfactory for the isolation of other serotypes. In practice, selenite and one other enrichment broth are used to cover the entire range of salmonella serotypes.

For many materials, particularly those in powder form which have

undergone heating during processing, e.g. dried egg products, gelatine, meat meals and fish meals, the use of enrichment media is unnecessary. Such products when suspended or dissolved in water, normal saline, or N/4 Ringer's solution form substrates capable of supporting the growth of any bacteria which they contain. For materials containing large numbers of *Pseudomonas* species, e.g. sewages, sewage effluents, polluted natural waters, the use of Jameson's (1961a) "secondary" enrichment is essential. Secondary enrichment consists simply of transferring a volume of the primary enrichment after 24 h incubation to a fresh tube of the same medium and incubating for a further 24 h before plating out. The method takes advantage of the difference in generation times between salmonellae (15–20 min) and pseudomonas (25–30 min) and allows a few salmonellae to outgrow within limits a much larger number of pseudomonas.

Quantitative Examination for Salmonellae

For some specimens, demonstration of the presence of salmonellae alone is required, while for others, an estimation of the Most Probable Number (M.P.N.) of salmonellae present is desirable. This examination is carried out by enriching a number of different volumes or quantities of the sample and noting the number of tubes positive at each dilution or quantity. The M.P.N. is then obtained by reference to tables. Many such dilution schemes have been designed for the examination of water samples and can be adapted to the examination of solid samples with little difficulty. Swaroop (1956) has discussed methods of attaining international comparability in the estimation of the bacterial density of water samples. His paper contains references to most of the dilution methods described.

For most purposes the tables given in McCoy (1962) are satisfactory.

Routine Methods

The following methods have been found useful in practice. Plate and enrichment media cultures are incubated at 37°. Enrichment broths are subcultured after 18–24 and 42–48 h incubation. Plates are examined after 18–24 h incubation. Single strength enrichment broths are used for all samples.

Qualitative

Human excreta

Plated direct on bismuth sulphite agar. At time of plating, portions of sample are emulsified in selenite F broth, and in one other enrichment broth. Enrichments are subcultured to bismuth sulphite agar.

Animal excreta

As for human excreta, omitting selenite F enrichment.

Animal tissues

To detect surface contamination, the samples are cultured in enrichment broth, without sterilization of surface and without maceration. To detect contamination of the bulk of the material, the sample is dipped into boiling water before maceration and addition to enrichment broth.

Quantitative

Using bismuth sulphite media, the numbers of salmonellae in a sample may easily be determined. The extra work involved is justified by the information obtained. In the diagnosis of human and animal infection it is enough to demonstrate the presence of salmonellae in excreta. The numbers present do not alter the diagnosis.

As a routine it is convenient to examine 100 g of an average sample of the material. Four 25 g lots of material are suspended in enrichment broth and plated after 18-24 h and 42-48 h incubation.

In general terms,

- 1 sample positive out of 4 indicates 1-2 salmonellae per 100 g of sample.
- 2 samples positive out of 4 indicates 3-5 salmonellae per 100 g of sample.
- 3 samples positive out of 4 indicates 5-7 salmonellae per 100 g of sample.
- 4 samples positive out of 4 indicates >10 salmonellae per 100 g of sample.

The Most Probable Number (M.P.N.) of salmonellae per 100 g of samples found to contain salmonellae is now determined by enriching 10 by 10 g lots of samples with 1, 2, or 3 twenty-five g lots positive. The range of numbers given by this series is from 1- >23. Ten 1 g lots of samples with four 25 g lots positive are enriched. This range extends from 10- >230.

Media

Selenite F. (Hobbs and Allison, 1945)

Tryptone or Polypeptone 5 g; mannitol 4 g; disodium phosphate 10 g; sodium acid selenite 4 g; distilled water 1000 ml. (Final pH about 7.0.)

If the medium is to be made and used immediately, only enough heat to dissolve the constituents is necessary. If stored before use, it is necessary to sterilize the medium either by filtration or by steaming for not longer than 30 min. The medium should not be autoclaved. Once sterilized, the medium keeps indefinitely.

Tetrathionate medium B. (Rolfe, 1946)

Iodine solution. Iodine 20 g: Potassium iodide 25 g: distilled water 100 ml
Thiosulphate solution. Sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) 25 g: distilled water 130 ml.

As a check on the accuracy of these stock solutions, 10 ml of the iodine solution should be titrated with the thiosulphate solution: about 19.5 ml of the thiosulphate solution should be required for complete neutralization.

Brilliant Green. 1% solution.

Nutrient broth. (1% Lemco, 1% Evans peptone.)

A half-inch layer of chalk is added to 100 ml of broth in 6 oz screw-capped bottles and the whole autoclaved at 121° for 20 min.

When cool, to each bottle containing 100 ml of broth is added 0.1 ml brilliant green, 7.0 ml iodine solution, 14.0 ml thiosulphate solution. The bottle is shaken vigorously and poured into sterile tubes in 8–10 ml quantities. Each batch of tubes should be tested to ensure the absence of free iodine.

This medium contains the minimum of free thiosulphate and it is important that the volumes of iodine and thiosulphate solutions should be measured accurately. Medium B allows the growth of few organisms other than salmonellae.

Magnesium chloride/malachite green medium. (Rappaport *et al.*, 1956)

Stock solutions. A. Bacto tryptone 0.5 g: NaCl 0.8 g: KH_2PO_4 0.16 g: bi-distilled water 100 ml.

B. 40 g MgCl_2 (Analar) is dissolved in 100 ml water. As this salt is hygroscopic, it is advisable to dissolve the entire contents of a freshly opened container.

C. 0.4% solution of malachite green in distilled water.

For use, to each 100 ml of solution A, 10 ml of solution B and 3 ml of solution C is added. The final medium is distributed in 5 ml quantities in test tubes, autoclaved at 115° for 10 min, and stored in a refrigerator.

Modified bismuth-sulphite agar

The basal medium contains: Lab. Lemco 30 g; peptone 50 g; agar 120 g; ferric citrate scales 2 g; brilliant green 1% (w/v) 5 ml; distilled water 5000 ml.

The ingredients are dissolved by heating in the steamer, the solution is bottled in 500 ml amounts and autoclaved for 20 min at 115° . An indicator is also prepared, containing bismuth ammonia-citrate scales 3g; sodium sulphite (anhydrous) 5 g; di-sodium hydrogen orthophosphate (anhydrous)

5 g; dextrose 5 g; distilled water 100 ml. The mixture is brought to the boil, allowed to cool to room temperature, and added to 1 litre of the basal medium at 55°. The medium is poured into Petri dishes in a thin layer.

If large quantities of the medium are used, considerable economy can be achieved by substituting yeastrel 4 g for Lab. Lemco. This necessitates increasing the sodium sulphite (anhydrous) in the indicator solution to 10 g.

The Identification of Salmonella Serotypes

Salmonellae in pure culture on a primary plate or on a plate from enrichment may be picked off direct for slide agglutination. Single colonies from plates crowded with other organisms may be picked off with a straight wire under a plate microscope and inoculated on to slopes of nutrient agar to which a drop or two of nutrient broth has been added just before inoculation.

The slopes are incubated in a water bath at 37°. After 4 h incubation there is usually sufficient growth on the slope and in the broth at the foot of the slope for direct slide agglutination with polyvalent "O"; polyvalent "H" specific and non-specific salmonella sera; and *S. typhi* Vi serum (Memorandum, 1961). 1051 serotypes have been described (Kelterborn, 1967). Polyvalent salmonella "O" serum contains antibodies sufficient to agglutinate with 660 of these. Polyvalent salmonella "H" serum contains antibodies to all but a few of these serotypes. For this reason, characteristic salmonella colonies which agglutinate only with polyvalent salmonella "H" serum should not be discarded but should be sent to the nearest Salmonella Reference Laboratory for identification.

The identification of "H" antigens is made much simpler by the use of Rapid Salmonella Diagnostic (R.S.D.) sera. R.S.D. sera, however, do not contain the "H" antigen "i", the first phase antigen of *S. typhi-murium* which must be tested for separately. As *S. typhi-murium* is the most common serotype met in man and animals throughout the world, screening all colonies picked for this antigen is highly rewarding.

Any laboratory isolating salmonellae should be able to identify the serotypes most commonly encountered in the United Kingdom and in imported materials.

It is not feasible for a routine laboratory to identify all the serotypes encountered. For confirmation of serotypes isolated and for the full identification of uncommon serotypes, strains should be sent to the nearest Salmonella Reference Laboratory.

The following range of sera (Burroughs Wellcome) has been found adequate for the routine laboratory.

Somatic antisera.

Polyvalent salmonella "O" groups A-G.

Sera for individual somatic 2; 3, 10; 4; 6, 7; 8; 9; Vi; antigens. 11; 13, 22; 15; and 19.

Flagellar antisera.

Polyvalent "H" specific and non-specific; polyvalent "H" non-sp. 1-7.

Rapid Diagnostic Sera 1, 2, 3.

Phase I (specific) a, b, c, d, E, G, i, k, L, r, y, z, Z₄Z₂₃, Z₁₀, Z₂₉.

Phase II (non-specific) 2; 5; 6; 7; x, Z₁₅, Z₆.

Use of agglutinating sera

Slope cultures from single characteristic colonies are screened against:

(a) Salmonella polyvalent "O", polyvalent "H" and Vi sera. Strains agglutinated by polyvalent "O" are then screened against the individual somatic antisera contained in polyvalent "O" serum. Strains not agglutinated by polyvalent "O" serum should not be discarded at this stage. These may contain antigens not represented in polyvalent "O" serum.

Strains possessing the "O" antigen 4 should be screened against "i" phase I specific "H" sera to identify *S. typhi-murium*, the most common serotype isolated.

(b) Strains are now screened against polyvalent "H" specific and non-specific, and against polyvalent "H" non-specific sera. If the strain is in the non-specific phase, screen against individual non-specific sera.

If the strain is in the specific phase, screen against Rapid Salmonella Diagnostic sera 1, 2 and 3, and read result according to the following table.

TABLE 1. Rapid Salmonella diagnostic H antisera

Agglutination with antiserum			Antigen present
1	2	3	
+	+	-	b
+	-	+	d
+	+	+	E
-	-	+	G
-	+	+	k
-	+	-	L
+	-	-	r

The serotype denoted by the symbol G is intended to cover the antigens g, m, and p.

That denoted by L should cover all types having the antigen l.

That denoted by E should cover all types having the antigen e. If E is indicated, try non-specific sera enx, and enz₁₅ as E may be found in either phase.

If the organism is in the specific phase and does not react with R.S.D. 1, 2 and 3 sera, try a, c, y, z, z₆, z₁₀, z_{4z23}, and z₂₉.

Phase reversal

Complete identification of a serotype requires that both phases be identified. Phase reversal is effected by placing a few drops of the Phase serum which agglutinated the strain in a molten Craigie tube cooled to 50°. The serum is well mixed with the molten agar, and the tube allowed to cool. When the agar has set, the strain is inoculated into the centre tube. After incubation at 37°, growth is harvested from the medium outside the centre tube and agglutinations are carried out with the relevant individual "H" sera.

This method is rather lavish in the use of sera. In laboratories where many strains are isolated, Jameson's (1961*b*) filter paper method of phase reversal is more economical in sera.

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Methods for the Detection of Salmonellae in Meat and Poultry

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Many techniques and media have been described for the isolation of salmonellae from foods. At the present time no single simplified system of techniques has received international acceptance. Many systems are complex and time-consuming.

The system of techniques described in this chapter is intended to point the way to a rather more streamlined means of detecting salmonellae, suitable for use in routine screening laboratories where large numbers of meat or poultry samples may have to be tested.

The sample preparation techniques employed for salmonella testing have to be modified depending on the nature of the food material under investigation. This chapter describes in detail the preparation and enrichment of meat and poultry samples, but much of the material will also be of interest to those testing other types of foods.

The isolation methods described are based on fairly conventional culture principles. The fluorescent antibody technique and its use in salmonella detection has been given in detail elsewhere (Georgala and Boothroyd, 1964; 1965*b*). Formulation of media and description of their preparation and use are included at the end of this paper.

Principle of Test Systems

The salmonella detection system described here involves selective enrichment of the sample for 1 and 2 days, at which times the enriched sample is streaked on to a selective diagnostic agar. Suspect colonies are identified by biochemical and serological tests (Fig. 1).

Selenite enrichments are incubated at 43° instead of 37°, permitting the successful use of a "mildly" selective agar (brilliant green agar—BGA), without a second selective agar. The tests for confirming the identity of suspect colonies have been cut to a minimum, yet will usually ensure rapid and reliable recognition of salmonellae.

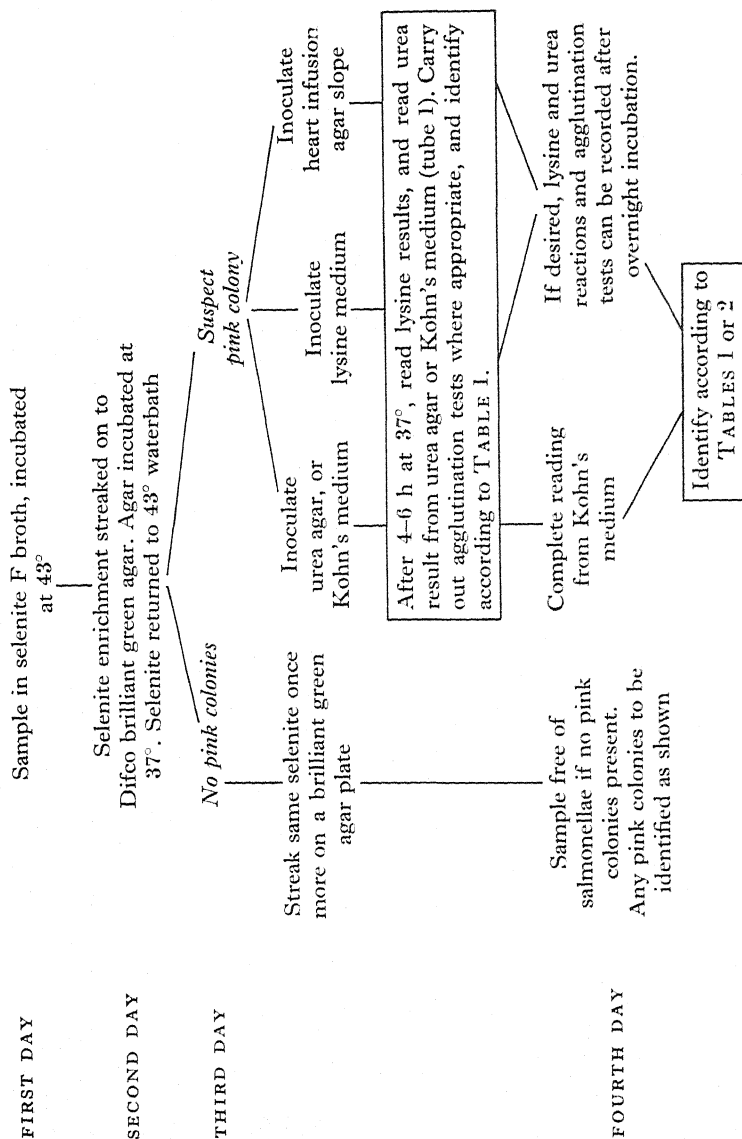


FIG. 1. Scheme for Salmonella testing of meat and meat products

The reasons for this choice of techniques, and the rationale of 43° incubation of selenite enrichments have been discussed by Harvey and Thomson (1953) and Georgala and Boothroyd (1965a).

Experience has shown that non-selective pre-enrichment is not required when testing raw or frozen meat or poultry products, therefore pre-enrichment techniques are omitted.

Sample Size and Preparation of Enrichments

The enrichment treatment given a sample depends on the nature of the sample. For example, contamination on carcass meat and poultry is usually surface located, while in comminuted meat products, sausages, etc., the salmonellae may be anywhere in the product. Comminuted meat products (but not carcass meat and poultry) therefore require homogenizing to expose the salmonellae to the enrichment medium.

For adequate recovery of salmonellae from contaminated food materials, samples of at least 25 g must be tested. This sample size is recommended here for carcass meat, boned meat, poultry, etc. A sample size of 50 g is recommended for comminuted meat products because these often consist of a mixture of meat and other materials.

The following enrichment techniques are based on these considerations:-

Carcass meat, boned meat, etc.

Cut the 25 g sample into about 10-15 small pieces with sterile scalpel, scissors and forceps. Weigh directly into a 250 ml sterile conical flask with cap, using a beam balance. Add 100 ml single strength Leifson's selenite F broth to each flask.

Dressed poultry

Obtain the 25 g sample by taking pieces of skin and underlying meat from various parts of the carcass. Include as much skin as possible. (There is evidence that the contamination on giblets in dressed poultry may not be representative of the contamination on the carcass itself.)

Comminuted meat products

Highspeed mechanical homogenizing is recommended. For salmonella testing the homogenizer cups must be very carefully sterilized to avoid cross-contamination between samples.

Homogenize the sample according to one of the following procedures:-

a. Weigh 50 g of sample into a sterile homogenizer cup. Add roughly 200 ml of selenite broth to the sample from a flask containing 400 ml sterile single strength selenite F broth. Homogenize the sample at top speed for 2 min. Return the homogenate to the flask containing the remaining 200 ml selenite broth. Shake before incubating.

b. Homogenizing in 0.1% peptone broth is necessary when the homogenate is also to be used for other determinations, e.g. total viable counts, staphylococcal counts, etc. Whatever total volume of homogenate is prepared, the volume enriched for the salmonella test must contain at least 50 g of sample. This is added to an equal volume of *double strength* selenite F broth in a suitable flask. The final mixture should be 50 g of sample in a total enrichment volume of about 500 ml.

Meat factory and abattoir effluents

Wherever possible use sewer swabs, made of large pieces of an absorbent material such as cheesecloth or absorbent gauze. Fold the pieces to give a 1.5–2 m strip about 10 cms wide, and 2–4 thicknesses thick. Staple or stitch the layers together. Attach 2 m pieces of string to the end of each strip. The swabs and attached string are rolled, packed in individual bottles, cans or paper wrappers, and sterilized by autoclaving at 121° for 15 min. The string of each swab should be so arranged that the sterile swab can be removed from its container without the fingers touching the cloth.

In use, the sterile swab is unrolled and lowered into the sewer, gully or drain under investigation, until the entire length of the cloth is drifting in the water. The attached string is secured to some nearby projection and the swab is left for 2–7 days. On recovery the wet swab is placed in a wide mouth flask, and 750 ml single strength selenite F broth is added.

Isolation of Salmonellae

All selenite F enrichments should be incubated at 43° in waterbaths accurate to $\pm 0.5^\circ$. After 18–24 h the flasks are removed for the first streaking on to Difco brilliant green agar and then returned to the waterbath for a further 18–24 h, after which they are streaked again. Thus two brilliant green agar plates are inoculated from each selenite enrichment culture.

The enrichment cultures are streaked on to brilliant green agar in such a way as to give the maximum number of discrete colonies. The inoculated brilliant green agar plates are incubated 18–24 h at 37°.

Pale pink colonies 1–2 mm in diameter are regarded as suspect salmonellae. They are usually surrounded by a pink zone in the agar. Yellow or green colonies (usually surrounded by yellow zones) are non-salmonella and are ignored (this procedure will of course miss the occasional rare lactose fer-

menting salmonella). Pink colonies showing definite flat spreading edges are *Proteus* spp. and can be ignored. However, if there is any doubt, carry out the recommended confirmatory tests on such spreading colonies. *Proteus* spp. split urea and will be rapidly detected in the Kohn's medium or on urea agar.

Brilliant green agar plates showing no pink colonies on first inspection should be left on the bench for 2-3 h and then re-examined. The occasional very pale pink colony will not be noticed at first, but will develop in colour and be readily visible at the second examination. Brilliant green agar plates should not be incubated longer than 24-28 h.

Confirmation of Suspect Salmonellae

The confirmatory tests described here are specially selected for use in conjunction with brilliant green agar. In particular, a positive lysine test from a suspect pink colony is very strong evidence that the organism is a salmonella. The other tests will help to increase the certainty of this identification, and are particularly useful in laboratories not having reliable polyvalent salmonella antisera. The confirmatory tests can be carried out as follows:-

In laboratories carrying out preliminary serological identification

If possible, test two or more suspect colonies from each brilliant green agar plate. Using a straight platinum wire inoculate each colony into the lysine decarboxylase medium, on to a moist heart infusion agar slope, and on to a urea agar slope. After inoculation each bottle of the lysine medium is layered with about 4-8 mm sterile liquid (medicinal) paraffin. Incubate these test cultures at 37°. The lysine decarboxylase and urease reactions will develop within 4-6 h, by which time the growth on the heart infusion agar slope will be adequate for slide agglutination tests with Poly O and Poly H salmonella antisera. Thus suspect colonies can easily be identified within a working day. Alternatively, the inoculated test media can be incubated 18-24 h and the reactions recorded on the following day.

The results of these confirmatory tests are interpreted as shown in Table 1.

In practice, almost all pink colonies will fall into category 1 or category 3. Category 2 (a) organisms are very occasionally isolated and require an extensive examination before they can be identified. Category 2 (b) organisms are also infrequent, but may well be salmonellae with O antigens not covered by the Poly O antiserum. It is, therefore, recommended that all isolates forming pink colonies on brilliant green agar and giving a positive lysine

TABLE 1. Biochemical and serological recognition of salmonellae from brilliant green agar (with serology)

	Category 1 Definitely salmonella	Category 2 Depending on antisera used maybe salmonella	Category 3 Definitely not salmonella
		(a)	(b)
Lysine decarboxylase (1)	+	+	+
Urease	—	—	—
Poly O agglutination	+	+	±
Poly H agglutination	+	—	+

(1) Lysine medium turns blue/purple when positive; yellow when negative

(2) ± = positive or negative reaction.

decarboxylase reaction should be carefully checked and if they cannot be positively identified should be sent to a typing laboratory.

In laboratories not possessing reliable antisera

As before, if possible, test two suspect colonies from each plate. The lysine decarboxylase test is again of prime importance. But, because no immediate serological confirmation is available, it is recommended that in addition Kohn's two tube composite medium be used. This demonstrates motility, urea breakdown, and fermentation of glucose, mannitol, sucrose and salicin.

Having inoculated the lysine medium, use a straight wire and inoculate the surface of the sloped tube 1 of Kohn's medium, then with the same inoculum stab the wire through the agar butt of the slope to the bottom of the tube. Kohn's tube 2 (a semi-solid agar) is then stab-inoculated from the same colony, again making sure the straight wire reaches the bottom of the tube. A single colony should supply ample inoculum for these two tubes, and also for the lysine decarboxylase test, provided a straight thin platinum wire is used. Incubate the Kohn's tubes for 18–24 h at 37°. The urea reaction (tube 1) can usually be recorded in 4–6 h.

The results of these tests should be interpreted as shown in Table 2.

Organisms in category 1 should be sent for typing. Only the occasional isolate should fall into category 2, and should, if possible, also be sent for further investigation at a typing laboratory.

General Comments on Salmonella Detection

1. If a single enrichment broth is used, selenite broth is probably best. Use of an additional enrichment broth greatly increases the number of subsequent operations, although it may only slightly increase the proportion of positive results.

TABLE 2. Biochemical recognition of salmonellae from brilliant green agar plates (without serology)

	Category 1 Definitely salmonella	Category 2 Possibly salmonella	Category 3 Definitely not salmonella	
			<i>Proteus</i>	
			(a)	(b)
Lysine decarboxylase	+	+	—	—
Kohn's tube 1	+(1)	not pink throughout	±	pink throughout
Kohn's tube 2	+	±(2)	±	±

(1) Kohn's tubes + = salmonella pattern, i.e. tube 1—yellow throughout, usually with gas bubbles in agar. Tube 2—does not change to yellow, and also shows organism is motile.

(2) Kohn's tube ± = colour unchanged, or turns yellow.

2. Incubation of selenite broth at 43° helps to suppress the high background flora of meat and meat products. For best results at 43°, use Leifson's selenite broth formulation (or Oxoid equivalent).

3. Difco brilliant green agar is a "mild" medium and gives rapid colony formation (salmonella colonies are always clearly visible after 18–24 h incubation at 37°). Difco brilliant green agar is easy to prepare and is now widely used in many countries.

4. Brilliant green agar may occasionally fail to reveal salmonellae, e.g. those rare strains that ferment lactose. Brilliant green agar used together with some other medium (e.g. the "harsher" desoxycholate citrate agar) will probably give highest overall recoveries, but a second plating medium is not usually necessary in an industrial routine screening laboratory. By using brilliant green agar alone, more samples can be tested than when using two plating media.

5. The techniques described here will not usually detect *Salmonella typhi*. Except in very exceptional circumstances, specific tests for *Salm. typhi* should not be carried out in food factories.

6. Salmonella detection in meat is usually a qualitative procedure. Most Probable Number counts can be carried out, but the salmonella counts on contaminated meat samples are frequently very low. However, the qualitative system described here can give some idea of the level of contamination if sufficient samples are tested and all samples are roughly of the same size. The results are then assessed on a frequency basis. For example, a batch of meat providing 20–60% positive samples is obviously carrying much greater contamination than another batch providing 0.5–1% positive samples.

7. The serological identification of salmonellae can only be relied upon if high quality antisera are available. Unfortunately, there is increasing evi-

dence that many commercial antisera are not of this quality. Greater reliance should therefore be put on the biochemical characteristics of the isolates. The ability to decarboxylate lysine is particularly important. A negative lysine test excludes almost completely the possibility that an organism is a salmonella (a few strains of *Salm. paratyphi C* are negative). Conversely, a lysine decarboxylase positive culture from a pink colony on brilliant green agar is very likely to be a salmonella, and requires careful testing with antisera of known reliability.

8. Although each enrichment is streaked on to brilliant green agar after 1 and 2 days incubation, suspect colonies on the second plate need only be examined if no confirmed salmonellae were found on the first brilliant green agar plate.

9. Colonies on brilliant green agar often lose their characteristic appearance on storage in a refrigerator. When such colonies cannot be removed for testing on the same day (e.g. over the weekend), mark their position on the Petri dish before placing in the refrigerator.

10. *Pseudomonas* strains can form pink colonies on brilliant green agar, and are occasionally a problem when testing raw or frozen poultry. However, pseudomonads are oxidase positive (salmonellae are negative) and this characteristic can be easily determined by transferring part of a colony to a suitable test paper (commercial oxidase test papers are available).

11. *Proteus* colonies on brilliant green agar are easily recognized by the spreading skirts around the colonies. If heavy *Proteus* contamination is encountered, sulphadiazine can be added to the brilliant green agar at 80 mg sodium sulphadiazine per litre of agar. The sulphadiazine selectively inhibits *Proteus* colony formation.

12. If agar slopes and bottles of lysine medium are pre-heated to 37° before inoculation, more rapid growth will take place after inoculation, and test results will be available perhaps 1 or 2 h earlier (depending on density of inoculum).

13. Full serological typing of salmonellae usually calls for analysis of two flagella phases. The Jameson paper strip technique is an outstandingly successful and economical means of performing flagella phase changes (Jameson, 1961).

Media for Salmonella Detection

Selenite F Broth—Single strength (Leifson, 1936)

Sodium acid selenite	4 g
Peptone (Evans)	5 g
Lactose	4 g
Disodium hydrogen phosphate (Na_2HPO_4)	9.5 g

Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$)	2.5 g
Distilled water	1000 ml

Final pH 6.9-7.1

The solid ingredients are stirred into the water, which should be pre-warmed to about 60°. If the ingredients are weighed carefully, the pH before sterilizing will be about 7.1, and requires no adjustment. If pH adjustment is necessary, this should be done with one of the phosphates.

Bottle the medium and sterilize by steaming for 30 min.

Special notes

- Oxoid selenite broth has been found an acceptable commercial substitute for the above medium.
- After steaming, selenite F broth should contain a slight suspension of selenium, which gives it a pink opalescent appearance. On standing this will settle as a fine red deposit.
- Steaming selenite broth does not always ensure sterility, particularly with large volumes. If the medium is to be kept for a week or longer before use, store in the refrigerator.

Selenite F Broth—Double Strength—Make as if single strength, but with double the quantity of ingredients.

Brilliant Green Agar—Difco brilliant green agar may be used. The stock agar can be bottled in 100 ml volumes in medical flat bottles, and stored at room temperature in a dark place. When required, melt the agar by steaming, cool to about 50° and pour about 7 plates (9 cm diameter) from each 100 ml medium. Sterilize exactly as recommended in the Difco Manual (121° not exceeding 15 min), and when melting the bottled agar use the shortest heating time possible.

Brilliant green agar is fairly sensitive to bright light. Bottles and plates of the medium should not be left in direct sunlight at any time during preparation and use, and for storage for longer than a day should be kept in a dark place.

Lysine Decarboxylase Medium (Moeller, 1955)

Peptone (Difco)	5 g
Meat extract (Oxoid Lab-Lemco)	5 g
Glucose	0.5 g
Bromocresol purple (1.6% stock solution)	0.625 ml
Pyridoxal hydrochloride	5 mg
DL-lysine hydrochloride	20 g
Distilled water	1000 ml

Adjust final pH to 6.0

22080

Dispense in about 3–4 ml quantities in small screw-cap (Bijou) bottles, and sterilize by autoclaving at 121° for 15 min.

Reaction

A positive lysine decarboxylase reaction changes the medium to blue/purple; a negative reaction changes the medium to yellow (the indicator in the medium described here differs from that recommended by Moeller, 1955).

Special notes

a. Inoculated bottles of the lysine medium should be layered with 4–8 mm sterile liquid medicinal paraffin (the liquid paraffin can be bottled as 50 ml amounts in 100 ml bottles, and sterilized in a hot air oven at 160–170° for 1 h).

b. If L-lysine is available instead of the DL variety, use 10 g per litre instead of 20 g.

c. When this medium is made for the first time, also prepare a control batch without lysine. Bottle and sterilize in the same way and label clearly. Then, to begin with, always inoculate each suspect culture into a bottle of test (with lysine) and control (without lysine) media. Cover both with a layer of sterile liquid paraffin. A lysine decarboxylase positive culture will cause the test medium to change to blue/purple and the control medium to yellow; with a lysine decarboxylase negative culture both media will change to yellow. When it is seen that the test is working satisfactorily, dispense with the control medium, and only use the test lysine-containing medium.

Heart Infusion Agar—Difco heart infusion agar gives more luxurious salmonella growth than most nutrient agars, and is therefore ideal for agglutination tests on young cultures. If possible, prepare slopes in screw cap bottles and after sterilizing make sure the caps are tightly closed. This will ensure that the medium remains moist, a necessary condition for rapid growth and successful formation of flagella.

Urea Agar—Oxoid or Difco urea agar base media can be used. The urea component of these media cannot be sterilized by heat. For this reason Oxoid supply filter sterilized urea in ampoules, which can then be added to the Oxoid urea agar base after the base has been autoclaved and cooled. Alternatively, 20% or 40% urea solution can be sterilized by Seitz filtration, and added, as required, to the heat sterilized Oxoid base (equivalent to 20 g solid urea per 1000 ml base). The Difco urea base contains the urea, and has to be Seitz-filtered before the addition of heat sterilized agar solution. On both of these media *Proteus* spp will produce a pink colour (ammonia from urea) within 3–6 h, and will turn the medium completely pink/red after overnight incubation. *Salmonella* produce no ammonia from urea,

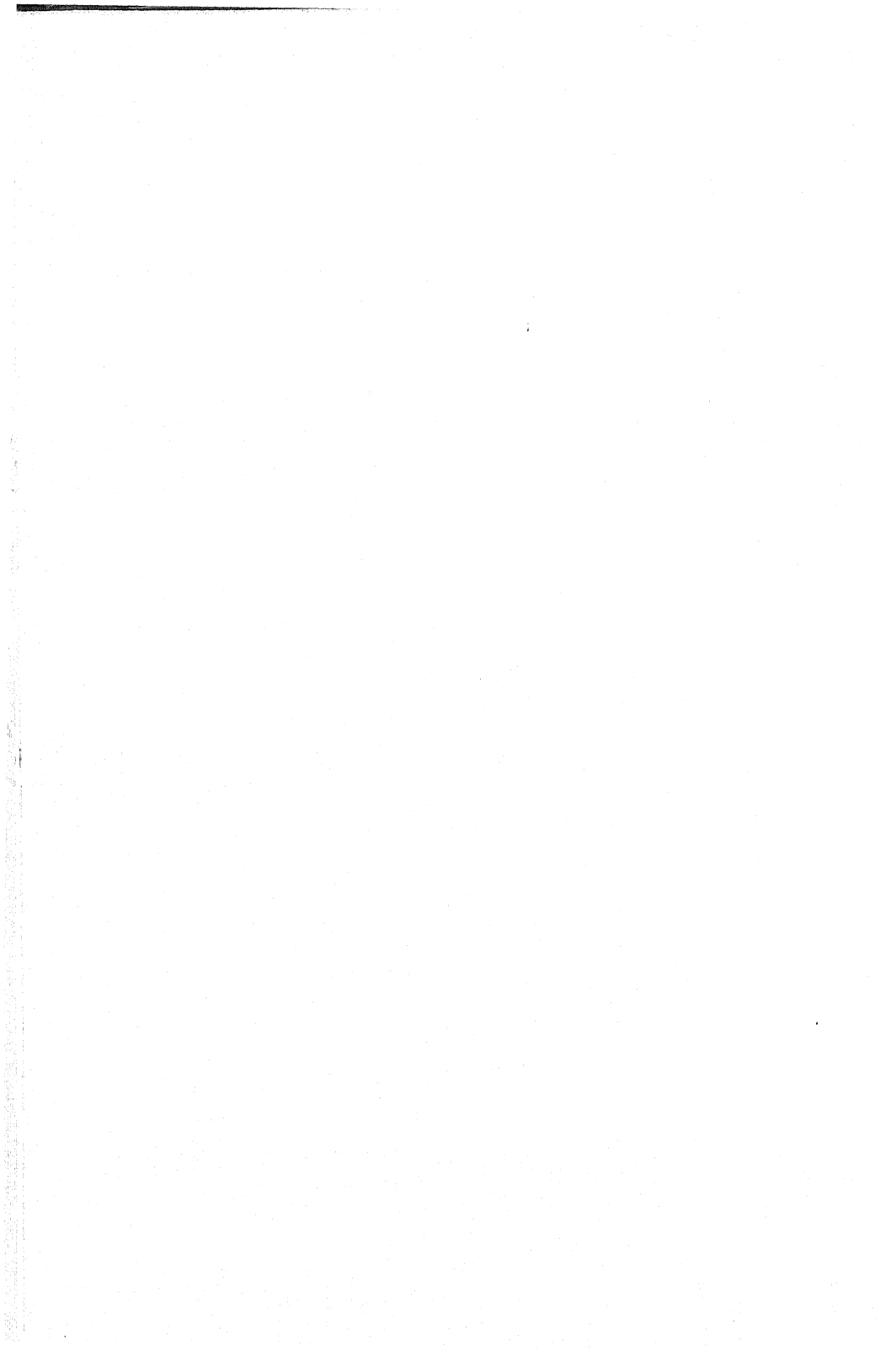
and the medium therefore retains its neutral colour, or turns slightly yellow.

Kohn's Two Tube Composite Medium—Two different media, are used in separate tubes. The Oxoid version of the medium is useful. Medium 1 is prepared in the form of slopes with fairly deep butts. Medium 2 is a soft agar solidified in an upright position. Both tubes are inoculated with a straight wire. Tube 1 is inoculated on its sloped surface, and stabbed into the depth of the butt. Tube 2 is stab-inoculated to the bottom of the tube. Consult the Oxoid manual before using this medium. Except for the urea reaction (incorporated in tube 1), both the tubes require overnight incubation at 37° for clear demonstration of the various reactions. The urea reaction (tube 1 turns pink) is usually visible within 3–6 h.

An improved version of this medium was reported by Gillies (1956). The Gillies version has a complicated formula but gives very clear cut reactions, and therefore can be recommended.

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Isolation Methods for Mycoplasmas from Man and Rodents

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Thirty years ago, mycoplasmas from rodents were first isolated and recognized as mycoplasmas (then known as pleuropneumonia-like organisms or PPLO). In 1937, mycoplasmas were first isolated from man and since then there have been many similar reports. However, classification of these mycoplasmas was not put on the systematic basis now recognized until the work of Edward and Freundt (1956). The first unequivocal human pathogen *M. pneumoniae* (Eaton agent) was not grown on non-living media until 1962 (Chanock *et al.*, 1962a) because only then were its fastidious growth requirements recognized. The isolation of this organism stimulated a great deal of new interest in mycoplasmas amongst workers in many fields including veterinary medicine in which mycoplasmas have, for many years, been recognized as important pathogens. However, despite all the work done since the *M. pneumoniae* was first isolated, no new pathogenic human species have been discovered although several new commensal species have been characterized. Much confusion has arisen due to work with tissue cultures, especially in the case of continuous cell lines. These are frequently found to be contaminated with mycoplasmas, and hence any supposed isolation of mycoplasmas using tissue culture techniques must be open to serious doubt. For this reason the isolation methods to be discussed do not include tissue culture.

The mycoplasma species isolated from man which have so far been characterized are listed in Table 1.

Those isolated from rodents are listed in Table 2.

In addition to the species, listed in Tables 1 and 2 there are others not yet named (e.g. Navel), and undoubtedly others will be described.

Specimens for Isolation

In common with any specimen for isolation of micro-organisms ideally there should be as little separation in terms of time and intermediate carriers, such as swabs, between the tissue and the culture media. As rodents are usually laboratory animals the problems should be minimal. Where

TABLE 1. *Mycoplasma* species isolated from man

<i>Species</i>	<i>Site</i>
<i>M. fermentans</i>	Genito-urinary tract
<i>M. hominis</i>	Genito-urinary tract Pelvic abscesses (Salpingitis) Blood culture
<i>M. laidlawii</i> <i>M. orale</i> type 1 (Syn <i>M. pharyngis</i>) <i>M. orale</i> type 2 <i>M. orale</i> type 3	Oro-pharynx
<i>M. pneumoniae</i>	Respiratory tract (Blister fluid in erythema multiforme)*
<i>M. salivarium</i>	Oro-pharynx
T-strain mycoplasmas	Genito-urinary tract Oro-pharynx

* (A. M. Gordon—personal communication.)

TABLE 2. *Mycoplasma* species isolated from rodents

<i>Species</i>	<i>Animal</i>	<i>Site from which usually isolated</i>
<i>M. arthritidis</i>	Rat	Joints
<i>M. neurolyticum</i>	Mouse	Brain, conjunctiva, respiratory tract
<i>M. pulmonis</i>	Rat	Respiratory tract, middle ear
	Mouse (Rabbit)	

there is obvious disease, the pus or ground-up infected tissue is the inoculum of choice. Where there is no obvious disease—e.g. in checking a rodent colony for inapparent mycoplasma infection—a swab from the nasal turbinates or middle ear cavity would be appropriate. In man the patient is usually at some distance from the laboratory and swabs of pus, pharyngeal or vaginal secretions, or, in the case of respiratory infection, sputum, are the usual specimens. These specimens should reach the laboratory as soon as possible. If overnight storage is unavoidable this should be at 4° (Andrews *et al.*, 1967). *Mycoplasma* medium may be used as a transport medium if

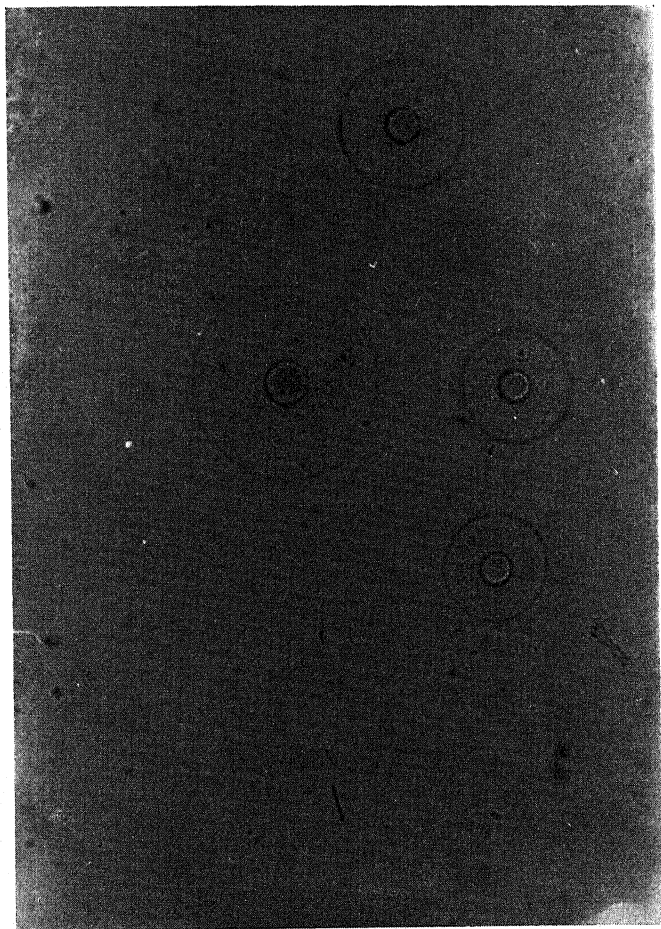


FIG. 1. Typical mycoplasma colonies on Hayflick's medium. Viewed by transmitted light they show classical "fried egg" appearance. X 37.5

the specimen has to travel some distance and if there is a danger of it drying in transit. Csonka *et al.* (1966) report the isolation of T-strain mycoplasmas and *M. hominis* after up to three days storage of urethral material in Stuart's transport medium. As yet there has been no *critical* study published of the best type of swab for transport of mycoplasmas in clinical specimens.

Media for Isolation

Until the isolation of *M. pneumoniae* various media had been used for the isolation of mycoplasmas. However, the medium devised by Hayflick

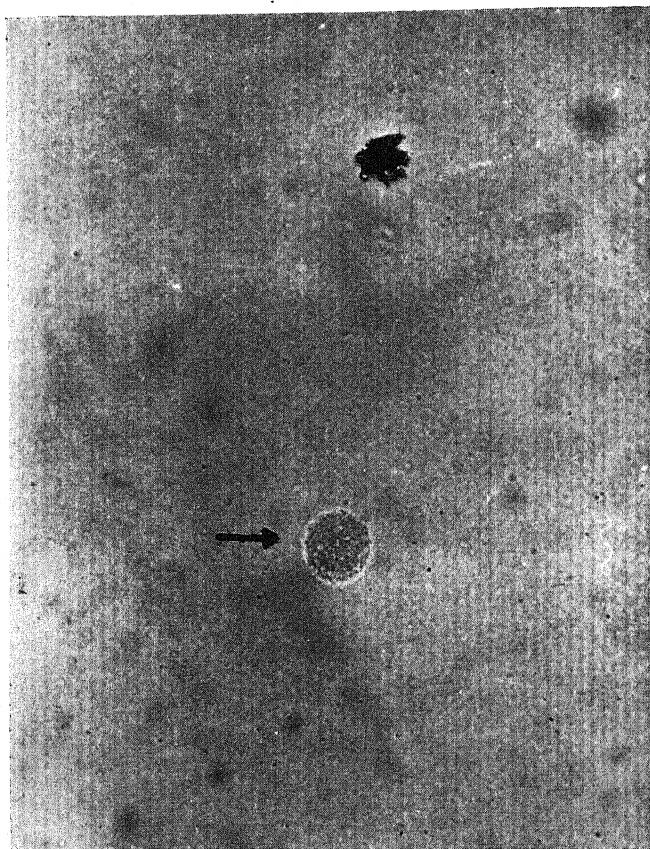


FIG. 2. A colony of *M. pneumoniae* showing its typical reticulated appearance. Note the absence of a typical peripheral zone unlike that shown in FIG. 1. X 166.

(Chanock *et al.* 1962*a*; Chanock *et al.*, 1962*b*) was not only the first medium on which *M. pneumoniae* grew but also supports the growth of all human and rodent mycoplasmas listed in Tables 1 and 2. Typical mycoplasma colonies on Hayflick's medium are shown in Fig. 1. Figure 2 shows a colony of *Mycoplasma pneumoniae*. This medium, with some variations, is now in wide use. The recipe for the basic medium is given below:-

PPLO broth or agar without crystal violet (Difco)	7 parts
25% yeast extract	1 part
Unheated horse serum	2 parts
Benzympenicillin	1000 units/ml
Thallous acetate	1/2000 final concentration

The PPLO broth or agar is made up according to the manufacturers' instructions and following sterilization at 121° for 15 min, may be stored in suitable amounts. Yeast extract is made up as follows: 250 g of fresh yeast is added to one litre of distilled water which is then heated to boiling point. The mixture is filtered through two sheets of Whatman No. 12 filter paper and brought to pH 8 by the addition of NaOH. Centrifugation before filtration greatly speeds up this stage of the preparation as the yeast extract tends to filter slowly. The extract is dispensed in suitable amounts, autoclaved at 121° for 15 min and stored at -20°. Horse serum is usually stored at -20° until needed. Benzylpenicillin solution should be prepared freshly each time medium is made. Thallous acetate is conventionally prepared as a 2.5% solution and sterilized by Seitz filtration. 2 ml of this solution are added to each 100 ml of medium. Thallous acetate should be added before the horse serum otherwise clouding of the medium may occur (D. Taylor-Robinson—personal communication).

Modifications

Since this medium was described various modifications have been reported:-

a. Basic medium

Hayflick (1965) gave the following formula for the broth base:

Difco Beef Heart for Infusion	50 g
Difco Bacto Peptone	10 g
NaCl	5 g
Water	900 ml

Soak the Beef Heart for Infusion preparation for one hour in distilled water at 50°. Bring the solution to the boil and filter through two sheets of Whatman No. 12 filter paper. The peptone and NaCl are then added and the pH is increased to 7.8 by the addition of 1.6 ml of 10 N NaOH. The broth is boiled and again filtered. Finally it is made up to one litre with distilled water. 0.2 ml 10 N HCl is added before autoclaving at 121° for 15 min. The final pH should be 7.6-7.8 and the broth should be crystal clear.

b. Agar

Some workers have reported favourably on the substitution of Oxoid Ionagar No. 2 for Difco agar, and in the author's experience this appears to be quite satisfactory.

c. Yeast extract

Lemcke (1965) described a modification by Hers of the preparation of yeast extract.

1 Kg. of yeast is suspended in one litre of distilled water and is extracted at 80° at pH 4.5 for 30 min. The suspension is then centrifuged, the supernatant is removed, adjusted to pH 7.8 and sterilized by Seitz filtration. Lemcke noted that whereas colonies of *M. pneumoniae* on Hayflick's medium normally consist only of granular centres, on medium containing Hers extract the colonies presented the "fried egg" appearance typical of mycoplasmas growing under satisfactory conditions. House and Waddell (1967) reported a much higher recovery rate of mycoplasmas from tissue cultures using Hayflick's medium with Hers yeast extract as compared with the same medium using the normal, high temperature, yeast extract.

d. Serum

Some workers have found that swine or human serum may be substituted for horse serum. With human serum there is always the possibility of the presence of antibodies or even antibiotics so that media control (see p. 48) is of great importance.

e. Antibacterial agents

Ampicillin at a final concentration of 1 mg/ml of medium may be substituted for penicillin and thallos acetate. This is not only a simplification of the medium but avoids the use of thallos acetate which is toxic to T-strain mycoplasmas at a concentration of 1 in 2000.

f. pH

Although Hayflick's medium was originally described as being at pH 7.8, which is optimal for the isolation of *M. pneumoniae* and is satisfactory for some if not all rodent mycoplasmas, the optimal pH for isolating other human mycoplasmas is pH 6-6.5.

g. Additions to the medium

Some workers add 20 µg/ml calf thymus deoxyribonucleic acid to the medium whilst 1% dextrose in fluid medium will enhance the growth of *M. pneumoniae* in stationary cultures (Low and Eaton, 1965). Tauraso (1967) reported that DEAE dextran (10 mg/100 ml of medium) enhanced the growth of some mycoplasma species on agar medium, apparently by binding inhibitors present in commercial agar preparations.

For a valuable review of media used for the growth of mycoplasmas see Lemcke (1965).

Special media

These fall into two groups:-

- (a) Media for the isolation of T-strain mycoplasmas.
- (b) Selective medium for *M. pneumoniae*.

a. T-strain media.

Although T-strain mycoplasmas may be isolated on Hayflick's agar medium at pH 6 they also can be grown very satisfactorily on other media. A medium specifically designed for the isolation of T-strain mycoplasmas has been described by Shepard (1967). The medium designated A2, has the following composition:

Trypticase Soy Broth Powder (Baltimore Biological Laboratories) 30 g.
Distilled water, 1 litre.

(For solid medium add "Oxoid Ionagar No. 2" 11.53 g)

Bring to pH 6 with N. HCl.

Bottle in 76 ml amounts. Autoclave at 121° for 15 min.

For use add 20 ml of unheated horse serum (brought to pH 6 with 1N HCl), 5 ml (3 ml for agar medium) of yeast extract (made by boiling a 25% suspension of baker's yeast in distilled water for 2 min clarifying by filtration, bringing to pH 6 and autoclaving at 121° for 15 min) and 1000 units of benzyl penicillin per ml. If trypticase soy broth powder is not available Shepard (1967) has described in detail the preparation of the medium from basic ingredients. Presumably, ampicillin may be substituted for benzyl penicillin here and the wider antibacterial spectrum of this antibiotic will increase the efficiency of the medium for primary isolation purposes.

The addition of 0.1%–1% urea to the solid medium results in the formation of slightly larger colonies which have a yellow to brown pigmentation.

A useful indicator broth was described by Purcell *et al.* (1966). This consisted of Hayflick's fluid medium plus 0.1% urea and 0.002% phenol red, the medium being at pH 6. T-strain mycoplasmas split urea to form ammonia. The resulting pH change causes the medium to change from yellow to red. In the absence of contaminating bacteria such a colour change is highly suggestive of the presence of T-strain mycoplasmas. Ideally subcultures to solid medium should be made before the medium turns red as at alkaline pH the T-strain mycoplasmas die very rapidly.

b. Selective medium for M. pneumoniae

A selective medium for *M. pneumoniae* was described by Kraybill and Crawford (1965) who observed that methylene blue was inhibitory to human-associated mycoplasmas other than *M. pneumoniae*. This medium was modified by Smith *et al.* (1967) and is distributed in either Bijoux bottles or 1 dram screw cap vials. A thin-layer of Difco PPLO agar (or agar base made from Difco PPLO broth and Ionagar No. 2 as previously described) is poured into each vial. When the agar has set, 2 ml of Hayflick's broth containing 1% dextrose, 0.001% methylene blue and 0.002% phenol red is added. *M. pneumoniae* reduces the methylene blue and produces acid

from the dextrose so that positive cultures become pale green. (Other workers have used 0.003% methylene blue and 0.004% phenol red with success.) It should be mentioned here that some mycoplasmas e.g. *M. hominis* will grow on a good horse blood agar as tiny colonies which may be seen with a hand lens. They may well occur in the zone of inhibition around a penicillin or ampicillin sensitivity disc where a direct antibiotic sensitivity test has been set up on a blood agar plate inoculated directly with the specimen under examination. More sophisticated media used successfully for the isolation of mycoplasmas from human bone marrow have been described by Barile *et al.* (1966), and Murphy *et al.* (1967).

Media Control

It is most important that any new batch of medium constituent should be examined in parallel with the previous batch for its ability to support the growth not only of stock strains of mycoplasmas but, if possible, of fresh strains isolated from infected material. Both commercial PPLO agar and horse serum may, on occasion prove to be inhibitory.

Conditions of Incubation

Most mycoplasmas grow better on primary isolation under anaerobic conditions rather than in air. For this reason it is essential that all cultures on agar medium should be incubated anaerobically. Aerobic incubation in parallel may be carried out but is less valuable than anaerobic incubation. The surface of agar media should be moist and should not be flamed in order to get rid of bubbles which may arise during plate pouring (D. Taylor-Robinson—personal communication).

Anaerobic incubation may either be in hydrogen, using a McIntosh & Filde's jar or in 90–95% nitrogen with 10–5% CO₂. Although the role of CO₂ is not yet clearly defined, aerobic cultures may also be incubated in an atmosphere containing 10% CO₂. Cultures should be incubated at 37°.

Confirmation of Identity of an Isolate as a Mycoplasma

Obviously this is a most important part of any isolation procedure particularly in view of the fact that not only is penicillin (which is a medium component) commonly used for producing bacterial L-forms but that L-form colonies may resemble mycoplasmal colonies very closely. The confirmation of identity of any isolate is based on the following criteria:-

Typical colonial morphology

Mycoplasma colonies must be differentiated from bacterial colonies and artefacts such as crystals on the surface of the medium (Hayflick, 1965) and platelet aggregates (Ryschenkow *et al.*, 1967).

Inability to scrape growth off agar media

The centres of mycoplasma colonies remain embedded in the medium when the growth is scraped with a loop. Colonies usually cannot be transferred to fresh agar medium using a loop.

Failure of subculture on antibiotic-free medium to reveal the presence of bacteria

Bacterial L-forms would be expected to revert to normal forms in early passage material (first passage if possible).

Failure to subculture on to serum-free medium

Human and rodent mycoplasmas require serum for growth.

Staining with Dienes' stain (Dienes, 1939)

Bacterial colonies decolourize the stain whereas mycoplasma colonies do not.

Inhibition of growth by specific antibody

It is important, as in diagnostic bacteriology, to obtain pure cultures before attempting to identify a mycoplasma. For this reason cloning of colonies either by plunging a straight wire into a colony and subculturing in broth or removing a plug of agar bearing a single colony with a pasteur pipette and placing it in broth and then subculturing from broth to agar medium and back to broth for up to 6 cycles, is most important. Obviously several colonies should be cloned and several of each type or size if there is variation in either of these features because cultures, especially from the respiratory or genito-urinary tract, may contain mixtures of mycoplasma. Growth inhibition tests using specific antisera may then be carried out (Clyde, 1964).

Subculture of Mycoplasmas

Mycoplasmas growing on agar medium cannot, as noted above, easily be subcultured like bacteria by merely transferring growth with a wire loop. To subculture to another agar plate, a block of the agar bearing colonies is cut out (this can easily be done with a wire loop) and placed face downwards on to the new plate. The block is then pushed over the agar surface and organisms are thus transferred to the new plate. Subculture to fluid medium is made either as above or by placing a block of agar bearing colonies into the fluid medium.

At all stages mycoplasma cultures should be handled with great care as the history of work with mycoplasmas contains many instances of cross contamination of cultures.

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Isolation of *Mycoplasma suipneumoniae*

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Pathogenic organisms of the mycoplasma group (formerly known as the Pleuropneumonia-like organisms or P.P.L.O.) are usually very fastidious in their growth requirements, and their isolation presents special problems. On solid medium, mycoplasmata form minute colonies (10–600 μ diam.), usually with a characteristic nipple-like centre embedded into the agar.

These organisms differ from bacteria in a number of important respects: most species of mycoplasmata require protein and sterols for growth; they lack a rigid cell wall and are therefore very pleomorphic. The delicate membrane surrounding the organism is triple-layered (by electron microscopy) and does not contain any cell-wall mucopeptide; thus mycoplasmata show an absolute resistance to penicillin. They are also resistant to 1:1000 thallium acetate, and their growth is inhibited by specific antibody. The smallest units capable of reproduction are less than 200 m μ diameter (i.e. they are as small as the larger viruses). Their method of reproduction is uncertain but it seems likely that they multiply by a process of complex budding.

Many of the pathogenic mycoplasmata have been isolated originally in association with living cells (e.g. in embryonated hens' eggs, tissue cultures or laboratory animals). But as these organisms are not yet identified on the basis of any common fundamental property, an isolate is not accepted as a mycoplasma until it has been cultivated as the characteristic colonies on cell-free solid medium. In practice, the development of transparent media that will allow the isolation, identification and passage of such colonies, has to be approached empirically, if a suspected mycoplasma does not grow on existing media. Almost certainly there are still important members of the mycoplasma group that have not yet been identified.

The type species of the group, *Mycoplasma mycoides* var. *mycoides*, which is the cause of the important disease contagious bovine pleuropneumonia, was first cultivated on solid medium as early as 1900. During the first 60 years of this century only a few pathogenic mycoplasmata were isolated—perhaps the best known being *Mycoplasma pulmonis*, the cause of respiratory

and middle-ear disease in laboratory rats and mice, and *Mycoplasma gallisepticum*, the cause of Nelson's coryza in chickens.

More recently there has been a great increase in the understanding of this group of organisms, which has largely stemmed from the successful cultivation on artificial medium of the human pathogen, *Mycoplasma pneumoniae* (Chanock *et al.*, 1962). Previously, Marmion and Goodburn, (1961), had suggested that the agent was probably a mycoplasma, because it was visible under the light microscope, (180–250 m μ in diameter) and because it was sensitive to streptomycin, the tetracyclines and organic gold salts. This agent had been isolated 20 years earlier by Eaton *et al.* (1942) and thought to be a virus.

Today there is a wide biological interest in this group of organisms: they are being studied fundamentally by molecular biologists because they are the smallest organisms known, which are capable of multiplying in cell-free media; they are of importance to workers using tissue cultures because mycoplasmata may accidentally contaminate these cultures or be mistaken for virus isolates e.g. from neoplastic tissues; they are of direct practical concern to medical, and particularly veterinary workers, since they are the causal agents of a number of economically important diseases. For a collection of recent papers covering many aspects of work on Mycoplasmata the reader is referred to "Biology of the Mycoplasma" (*Ann. N.Y. Acad. Sci.* (1967) **143**, Art. 1, 1–824).

My own experience in mycoplasmata has been in pigs, in which there is a common chronic pneumonia, worldwide in distribution and the cause of severe economic loss because affected animals are unthrifty. The earlier workers, studying the aetiology of this disease, now known as enzootic pneumonia, thought the causal agent was probably viral because of its filterability and resistance to penicillin and other antibiotics. It was first suspected in 1957 in both Sweden (Lannek and Wesslén, 1957) and in Britain (Whittlestone, 1957) that the causal agent was a mycoplasma. The first proof that this disease is caused by such an organism was published by Goodwin *et al.* (1965) in Britain. These authors were able to produce enzootic pneumonia with a mycoplasma that had been passaged on solid medium to such a high dilution (10^{-15} of the original material used to seed the first agar plates) that the mechanical transference of other agents was precluded. They named this enzootic-pneumonia-inducing organism *Mycoplasma suis pneumoniae*. Independently, in America, Maré and Switzer (1965) obtained mycoplasma colonies, which they named *Mycoplasma hyopneumoniae*, from liquid-media cultures which induced enzootic pneumonia. They did not provide any specific evidence that *M. hyopneumoniae* was the same as their pneumonia-inducing agent. Goodwin *et al.* (1967), however, showed that by the growth-inhibition and metabolic-inhibition tests,

M. suipneumoniae was serologically indistinguishable from the latter isolate. These authors also showed that, by the growth-inhibition test, *M. suipneumoniae* was unrelated to a wide range of recognized mycoplasmata and was therefore probably a new species. Currently, we are awaiting a decision from the Sub-Committee on the Taxonomy of Mycoplasmatales, as to which name should be used. For a description of *M. suipneumoniae* see Whittlestone (1967) and Goodwin *et al.* (1967).

The specific diagnosis of enzootic pneumonia of pigs is based on the isolation and identification of *M. suipneumoniae*. The isolation of the causal organism is of particular importance in this disease at the moment because antibodies have not yet been detected in the sera of pigs naturally affected with the disease.

The difficulties in isolating *M. suipneumoniae* and maintaining it in serial cultivation have been mainly responsible for the slow progress of research work on enzootic pneumonia. There are two main problems: first, *M. suipneumoniae* is fastidious in its growth requirements, and most recognized mycoplasma media do not support its growth. However, the organism can now be grown reasonably well in serial culture, both in cell-free liquid media (Goodwin and Whittlestone, 1964 and 1966; Goodwin *et al.*, 1965 and 1967; Maré and Switzer, 1965 and 1966), and on solid media (Goodwin *et al.*, 1965 and 1967). Second, mycoplasmata other than *M. suipneumoniae* are usually isolated from pneumonic-lung tissue collected from field outbreaks of disease thought to be enzootic pneumonia (Whittlestone, 1958; L'Ecuyer *et al.*, 1961; Dinter *et al.*, 1965; Switzer, 1967). In this context a common organism isolated is *Mycoplasma hyorhinis*, which grows more rapidly than *M. suipneumoniae*, and probably masks this latter organism. This problem has not yet been solved. In the author's experience, successful isolations of *M. suipneumoniae* have only been made by choosing enzootic-pneumonia lung from which other readily-growing porcine mycoplasmata cannot be cultivated on accepted mycoplasma media such as the one described by Edward (1947) but without bacterial inhibitors and with an added staphylococcal culture (Whittlestone, 1958).

Isolation Methods

Isolation in tissue cultures

The first successful isolation of *M. suipneumoniae* was made by Goodwin and Whittlestone (1963) in pig-lung tissue cultures, but what was probably the same organism had been cultivated earlier by Lannek and Wesslén (1957) and Betts and Whittlestone (1963).

A successful method we have used is as follows: inoculate healthy piglets (preferably piglets obtained surgically near term and reared in strict

isolation) intratracheally or intranasally with a suspension of enzootic-pneumonia lung. Kill the piglets 2-3 weeks later, collect sterile pneumonic tissue and prepare plasma-clot tissue cultures. The recipe for the feeding fluids for these plasma-clot tissue cultures and for the monolayer cultures is as follows:-

Pig serum (from enzootic-pneumonia-free pigs)	
inactivated at 56° for 30 min	20%
Lactalbumin hydrolysate	0.5%
Difco yeast extract	0.01%
Hanks' balanced salt solution (see Cruickshank 1965)	79%
Penicillin	200 units/ml
Mycostatin	50 units/ml

Incubate at 37°, changing feeding fluids as necessary, until the periphery of the cell sheets extending from the lung-tissue fragments start to show an increased cytoplasmic opacity and granularity, with accompanying acidity (compared with control cultures prepared from uninoculated piglets).

At this stage (usually 6-14 days after preparation of the cultures) transfer fluids from the plasma-clot cultures to partially-grown pig-lung-monolayer cultures in test tubes containing coverslips.

Two or three days after inoculation, remove coverslips from some of the lung-monolayer cultures (and their control uninoculated cultures), wash in warm phosphate buffered saline* (pH 7.2), fix for 2-5 min in methyl alcohol and stain with Giemsa stain (1:10 in phosphate-citrate buffer pH 7.2 for 1-3 h). Wash in two changes of acetone and two changes of a mixture of equal parts of acetone and xylol. Clear for 5 min in xylol and mount in D.P.X.

M. suis pneumoniae usually occurs as groups both supracellularly and away from the tissue culture cells. The organism is tiny and very pleomorphic, the commonest forms being cocci (0.5 μ diam. or less) and rings (0.6 μ -3.0 μ diam.). In the rings greater than 1.0 μ in diam. a single coccus-like dot (0.5 μ diam.) is usually seen. Sometimes the larger structures are associated with about 50 small cocci which are strung on very fine branching filaments (0.1 μ diam.—i.e. finer than the resolving power of the optical microscope, see Fig. 3).

The organisms occurring in tissue cultures may be specifically identified by the indirect fluorescent-antibody technique as described by Goodwin *et al.* (1967).

* The formula is as follows: Solution A; NaCl 8.0 g, KCl 0.2 g, Na₂HPO₄ 1.15 g, KH₂PO₄ 0.2 g, Water 800 ml. Solution B; CaCl₂ 0.1 g, Water 100 ml. Solution C; MgCl₂·6H₂O 0.1 g, Water 100 ml. Autoclave solutions A, B and C separately, at 110° for 10 min. Cool and mix aseptically.

Isolation in cell-free liquid medium

M. suipneumoniae was first cultivated in cell-free medium by Goodwin and Whittlestone (1964); from this basic medium *M. hyopneumoniae* has been transferred successfully to other media (Switzer, 1967). The basic medium has now been modified and improved (Goodwin *et al.*, 1965 and 1967). Compared with the medium used to feed the tissue cultures, this improved medium contains a different yeast extract, added lung broth or Hartley's broth and thallium acetate, but no mycostatin. The constituents are as follows:—

Lung broth or Hartley's digest broth (Lung broth autoclaved 112° for 30 min; Hartley's broth autoclaved 120° for 20 min)	30%
*Pig serum from enzootic-pneumonia-free pigs	20%
Lactalbumin hydrolysate (autoclaved 115° for 10 min)	0.5%
Hanks' balanced salt solution (autoclaved 120° for 10 min)	40%
*Yeast extract	0.05%
Penicillin	200 units/ml
*Thallium acetate (1:80 W/V in Elga deionized water)	1%

The lung broth is made by mincing enzootic-pneumonia-free pig lung with twice its weight of water, leaving overnight at 4° and filtering through double gauze. The filtrate is simmered for 15 min, filtered again through gauze and centrifuged (900 g for 20 min) and the supernatant autoclaved (112°, for 30 min) and tested for sterility.

The Hartley's broth is made in the usual way by digesting ox hearts with trypsin prepared from fresh pig pancreas. The enzootic-pneumonia-free pigs are obtained from herds checked in an enzootic pneumonia control scheme (Goodwin and Whittlestone, 1967). The yeast extract is prepared by extracting baker's yeast in its own weight of deionized water at 80° and pH 4.5 (by adding 38% HCl) for 20–30 min as described by Herderscheë (1963). The indicator is the phenol red present in the Hanks' salt solution which is made as described by Cruikshank (1965). The final pH of the medium is about 7.6.

The complete medium is stored at –25°. From each batch of medium, samples are tested for sterility and for their efficacy in supporting the growth of *M. suipneumoniae* by titrating deep-frozen, stock ampoules of the organism and by checking that the organism's growth is satisfactory during three passages.

M. suipneumoniae may be isolated directly from pneumonic lung in this medium, but we have used it mainly to cultivate mycoplasmas that have been previously isolated in tissue cultures.

*Sterilized by Millipore filtration.

In liquid medium, *M. suis*pneumoniae has a morphology similar to that occurring in the tissue cultures, except that the organism forms groups or colonies on the coverslips (Fig. 1), consisting of branching chains of cocci and globular structures of size range $0.3\ \mu$ – $16\ \mu$ (Figs. 2 and 3), (Goodwin and Whittlestone 1966; Goodwin *et al.*, 1967).



FIG. 1. *Mycoplasma suis*pneumoniae on coverslip in acellular medium. Groups and colonies. May-Grünwald-Giemsa ($\times 50$).

Isolation on solid medium

Solid media, which will support the continuous passage of colonies of *M. suis*pneumoniae, are described by Goodwin *et al.* (1965 and 1967). The first of these media contained the aqueous extract of normal pig lung; later this extract was replaced by Hartley's broth.

The recipe for the solid medium is the same as for the liquid medium described above, except that 1% of Oxoid Ionagar No. 2 is included. The agar is autoclaved with the Hanks' salt solution (at 124° for 15 min) and then cooled to 56° before adding the other sterilized constituents warmed to the same temperature. The plates are incubated at 37° in a moist atmosphere containing 5–10% CO_2 in air.

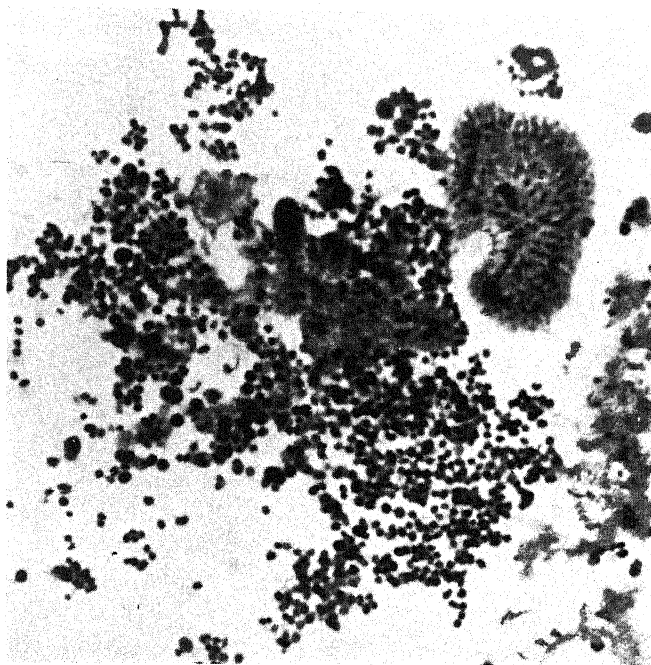


FIG. 2. *Mycoplasma suipneumoniae* on coverslip in acellular medium. Chains of cocci and globular structures of size range $0.3 \mu - 16 \mu$. May-Grünwald-Giemsa ($\times 2000$).

On these media *M. suipneumoniae* forms colonies $20 \mu - 100 \mu$ in diameter, detectable under the microscope after three days incubation, and some colonies reach a maximum diameter of 400μ at 7–10 days (Fig. 4). The older colonies show a slight central depression but we have not yet obtained colonies with the central nipple which is normally considered typical of the mycoplasma group. Giemsa-stained touch preparations of the colonies show mycoplasma-type pleomorphic elements with elementary particles about 0.3μ diameter.

These solid media have not yet been successfully used for direct isolation of this mycoplasma from enzootic-pneumonia lung, nor are there any reports of the use of any other solid media for the direct isolation of a mycoplasma of this serological type.

Solid-medium cultures are used, however, in the characterization of isolates as mycoplasmata, in their specific identification by the growth inhibition test, and in serial cloning of cultures prior to production of antisera in rabbits.



FIG. 3. *Mycoplasma suis pneumoniae* on coverslip in acellular medium. Detail of small colony showing cocci strung on fine branching filaments and a single large globular structure. May-Grünwald-Giemsa ($\times 3300$).

Criteria for the Isolation of *M. suis pneumoniae*

The criteria that we are using for the isolation of *M. suis pneumoniae* (see Goodwin *et al.*, 1967) are as follows:-

1. The presence in cell-free medium of a passagable acid-inducing factor.
2. An association between this factor and minute pleomorphic organisms of the morphology described above.
3. The development on solid medium, containing penicillin and thallium acetate, of small colonies composed of pleomorphic elements.
4. The inhibition of colony formation by the tetracyclines.
5. The failure to revert to bacterial colonies when passed in the absence of penicillin.
6. The serological identification of the organism using antiserum prepared against our original isolate from the J strain of enzootic pneumonia:
 - (a) by the metabolic-inhibition test using the method of Taylor-Robinson *et al.* (1966).
 - (b) by the growth-inhibition test of Huijsmans-Evers and Ruys (1956) as modified by Clyde (1964) and Stanbridge and Hayflick (1967).

7. For some isolates it is possible to test the ability of the organism to induce enzootic pneumonia in pigs, after it has been passaged in or on cell-free media to at least a 10^{-15} dilution of the original seed material.

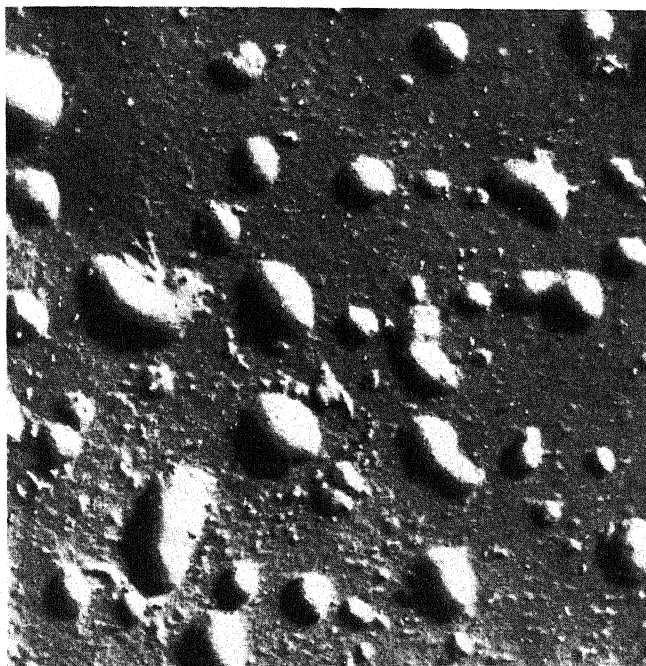


FIG. 4. Eight-day-old growth of colonies of *Mycoplasma suipneumoniae* on solid medium. These colonies were included in an enzootic-pneumonia-inducing inoculum ($\times 50$).

Future Studies

Several important problems on the isolation of *M. suipneumoniae* remain. The existing media or cultural methods for this organism could almost certainly be improved so that isolations of this organism could be made directly on solid medium. Clearly there is also the need for the development of selective media or cultural methods, that would allow the preferential isolation of *M. suipneumoniae* from material also containing other mycoplasmata. This may be possible on the lines described by R. J. Fallon elsewhere in this book, for the selective isolation of *M. pneumoniae* from man.

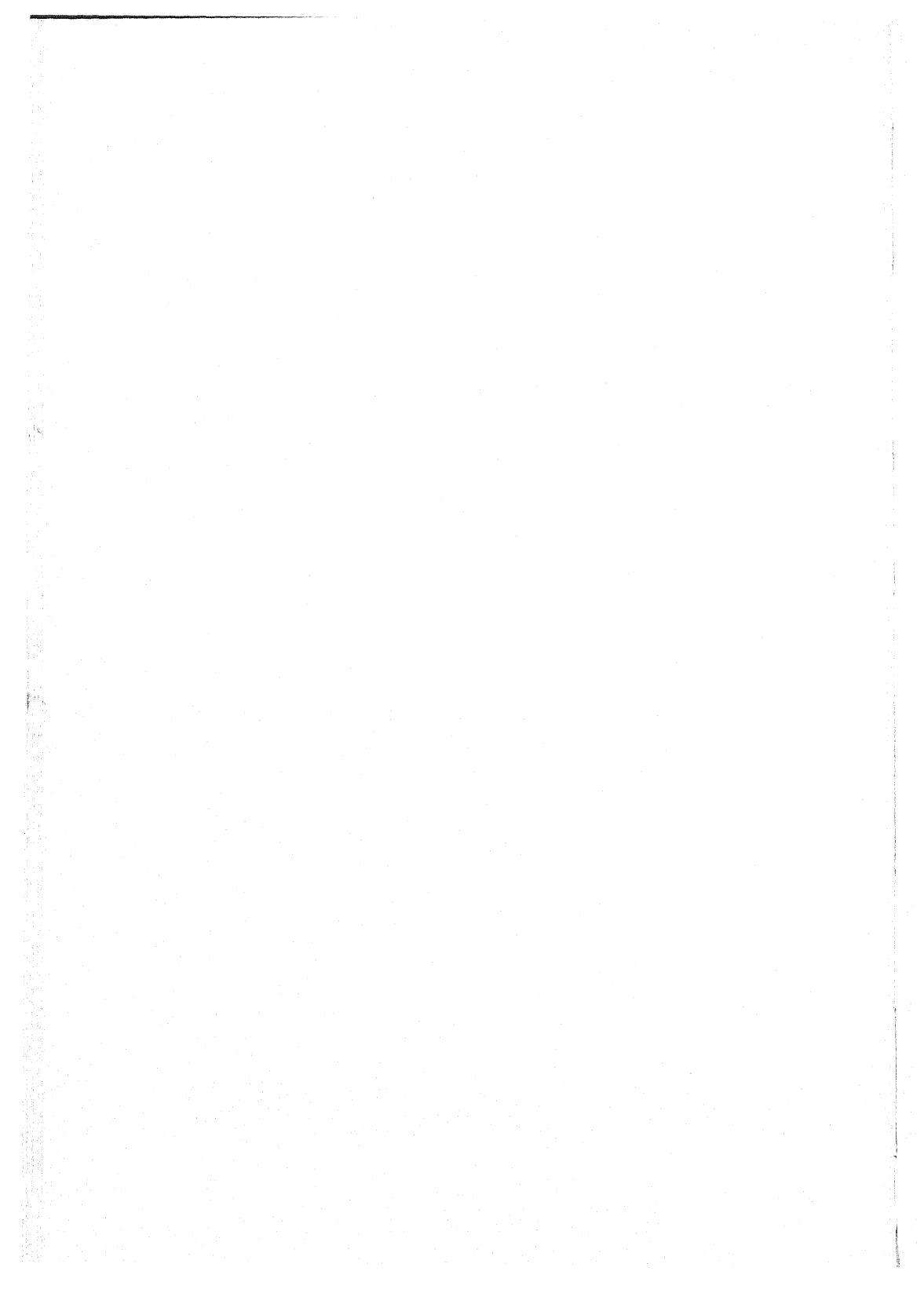
Acknowledgements

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Isolation of *Listeria monocytogenes*

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The causative organism of listeriosis, *Listeria monocytogenes*, was isolated and described by Murray *et al.* (1926). In *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957) it is classified as the sole member of the genus *Listeria* in the family Corynebacteriaceae. Larsen and Seeliger (1966) have proposed another species, *Listeria grayi*, which differs from *L. monocytogenes* serologically and by its ability to ferment mannitol.

The morphological, biochemical and serological characters of *L. monocytogenes* have been well described (for reviews see Seeliger, 1961; Gray and Killinger, 1966). There is still, however, a lack of knowledge about the mode of transmission and natural reservoirs of the organism. This stems from difficulties encountered in isolating *L. monocytogenes*. The difficulties of isolation were stressed by Murray *et al.* (1926) in their original description of the bacterium and later by Gill (1937). Because it is not easy to isolate, *L. monocytogenes* has come to be regarded as rare, and the sporadic occurrence of disease attributable to this species has encouraged this point of view. There is now evidence that *L. monocytogenes* is much more widespread than previously realized and that it does not always give rise to clinically acute infections (Seeliger, 1961; Gray and Killinger, 1966). It has been isolated from thirty-seven different species of mammals and from fish and crustaceans. It has also been isolated from water, sewage, soil, slaughterhouse waste, etc. Some workers think its natural environment may well be the soil (for discussion see Seeliger, 1961; Gray and Killinger, 1966). There is good evidence that listeriosis in sheep is caused by feeding on infected silage (Gray, 1960). Rappaport *et al.* (1960) established a relationship between listeria infections in apparently healthy women and repeated abortion. Pease (1967) has suggested a possible relationship between *L. monocytogenes* and auto-immune diseases in man.

The reason for the difficulties experienced in isolating *L. monocytogenes* is not understood. It has been suggested that it may exist as an L form in animal tissue (Pease, 1967). There is also evidence that a number of fungi and bacteria produce substances antibiotically active against *L. monocyto-*

genes and that some of these inhibitory substances are extremely stable in broth cultures (Larsen and Gundstrup, 1965; Larsen, 1966).

Isolation

A large number of methods have been proposed for the isolation of *L. monocytogenes*. Each has advantages and disadvantages and some appear to give better results in some laboratories than in others. An excellent review of the various techniques is given by Gray and Killinger (1966).

In our laboratory the most consistently positive results have been obtained using a combination of potassium thiocyanate enrichment (Lehnert, 1964); cold enrichment (Gray *et al.*, 1948); nalidixic acid-blood selective medium (Beerens and Tahon-Castel, 1966) and tryptose agar (Difco).

Using the procedure to be described *L. monocytogenes* was recovered from artificially infected soil, manure, grass clippings, slaughterhouse waste, sheep faeces and from milk and water. The material was infected with a small inoculum of *L. monocytogenes* and kept at room temperature in covered, but not sealed containers. Samples were taken on the day of infection and one, seven and fourteen days later. The solid materials were also sampled one month and three months later.

L. monocytogenes was also recovered from the brain of a calf which had been slaughtered at a local slaughterhouse after showing symptoms of incoordination of movement.

Media and methods

Potassium thiocyanate broth (Lehnert, 1964). Referred to subsequently as KT medium. Nutrient broth (Oxoid No. 2), 25 g; Tween 80, 1 g; potassium thiocyanate, 3.75 g; distilled water 1 litre; pH 7.4. Autoclaved 121° for 15 min.

Nalidixic acid-blood medium (Beerens and Tahon-Castel, 1966). Referred to subsequently as NAB agar. Nutrient broth (Oxoid No. 2), 25 g; nalidixic acid (Bayer), 0.04 g; distilled water, 1 litre; pH 7.4. Autoclaved 121° for 15 min. Nalidixic acid is dissolved in a small quantity (0.5 ml) of N NaOH and made up to required concentration with distilled water. Sterile horse blood (final concentration 5%) is added aseptically after autoclaving.

Blood nutrient agar. Blood agar base (Oxoid); Horse blood (final concentration 5%) added after sterilization.

Enrichment

Solid material

Brain and other animal tissue should be macerated with distilled water in a tissue blender. Soil, manure, silage, etc., may be added direct to the enrichment broth.

One to twenty gram samples are added to duplicate flasks of KT enrichment medium (100 ml). One flask is incubated at 4°, the other at 37°. After 24 h and 48 h incubation, 0.1 ml samples (or 0.1 ml of appropriate dilution, if much growth is evident in enrichment broth) from both flasks are plated, by the spreading technique, on to plates of blood nutrient agar (5% horse blood), NAB agar and tryptose agar (Difco). Incubate at 37° and examine after 24 h and 48 h. If negative results are obtained samples should continue to be plated from the enrichment broth held at 4° for periods up to three months.

Fluid material

Ten to fifty ml of a fluid sample (e.g. milk) is mixed with an equal volume of double strength KT medium. Incubation and subsequent procedure is as described for solid material.

Examination of cultures

The blood agar and NAB plates are examined for zones of β -haemolysis, after 24 h and 48 h incubation at 37°. After 24 h incubation on blood agar the colonies of *L. monocytogenes* are so small they are hardly discernable with the naked eye and can easily be missed.

The tryptose agar plates are examined microscopically with obliquely transmitted white light. Following the technique of Henry (1933) and Gray *et al.* (1948), the mirror is removed from a binocular scanning microscope and placed flat side up on the bench about 10 cm away from the base of the microscope which should have a glass stage. A white light source is so arranged that a beam of light at an angle of 45° is directed on to the mirror and reflected at an angle of 40°–45° through the glass microscope stage. A petri plate containing suspected *Listeria* colonies is placed on the stage and viewed ($\times 10$ or $\times 15$ magnification). The angle of light is important, direct transmitted light will not give the same results.

Material to be viewed in this way should be cultured on a clear, blood-free medium. The best results are obtained with tryptose agar (Difco). *Listeria* sp. colonies are quite distinctive when viewed in this way and with a little practice, can be recognized on a heavily contaminated plate. The colonies are very small, low convex, finely textured, bright blue-green in colour with an entire margin. Laboratory strains of *Listeria* sp. do not show the same bright blue-green colour but tend to be a much lighter green with the same finely textured surface. Gram-positive cocci, some *Bacillus* sp. and some coryneform bacteria are always present on all three plating media if the original sample is heavily contaminated, e.g. soil or manure. By obliquely transmitted light the colour and texture of colonies of these organisms on tryptose agar are quite different from those of *Listeria* sp.

Discussion

L. monocytogenes was recovered on every occasion from all the infected material. Reports in the literature indicate that while it is not difficult to reisolate *L. monocytogenes* from artificially infected material after 24 h it becomes increasingly difficult with time (Gray and Killinger, 1966).

L. monocytogenes was also recovered from the brain of the calf within 48 h of receiving the specimen.

The nature of the infected material determined whether the best results were obtained by incubation of the KT enrichment broth at 4° or at 37°.

L. monocytogenes was recovered with equal ease at the two enrichment temperatures in the case of water and milk. Higher counts were obviously obtained from the sample incubated at 37°.

Where the samples contained a more diverse flora, e.g. soil, manure, the best results were obtained when enrichment was conducted at 4°. There was a marked reduction of undesirable contaminants growing at this temperature.

In the case of calf brain tissue, *L. monocytogenes* was isolated from the enrichment broth at 37° but not at 4°. The organism was not recovered from either the refrigerated brain tissue or the brain tissue enrichment broth held at 4° even after one month.

The advantage of incubating the enrichment broths at 37° rather than at 4° is that results are obtained more quickly. This can be of great importance especially in clinical cases or where the source of infection is being sought, e.g. an outbreak of listeriosis in farm animals.

Many successful isolations of *L. monocytogenes* have been achieved with the cold incubation method of Gray *et al.* (1948) but in most cases a few weeks at least have elapsed before isolation was achieved. The cold incubation method is based on the ability of *L. monocytogenes* to grow slowly at 4° while most other micro-organisms do not grow at this temperature.

If, as shown by Larsen and Gundstrup (1965) and Larsen (1966) multiplication of *L. monocytogenes* is inhibited by substances secreted by other micro-organisms growing at a higher temperature, it is reasonable to suppose that the successful isolation achieved by using KT enrichment broth at 37° is due to the suppression of many contaminating micro-organisms. Our own results, and those of Lehnert (1964), indicate that potassium thiocyanate, at the concentration employed, is inhibitory for some *Bacillus* sp., some coryneform bacteria and almost all Gram-negative bacteria, with the exception of some *Proteus* sp. Gram-positive cocci are not inhibited.

In every case where *L. monocytogenes* was present it was detected on all three plating media. In our experience it was most frequently easier to detect on tryptose agar (Difco) plates using the Henry illumination tech-

nique (Henry, 1933), but often more *L. monocytogenes* colonies grew on blood agar medium. If the sampled material was heavily contaminated it was easier to detect *L. monocytogenes* on NAB medium because of a greater suppression of undesirable contaminants. Nalidixic acid inhibits all Gram-negative organisms tested with the exception of most *Pseudomonas* sp. and some *Proteus* sp. Most *Bacillus* sp. are also inhibited. Gram-positive cocci, some *Lactobacillus* sp., some coryneform organisms and *Erysipelothrix* sp. are not inhibited.

It is therefore recommended that as a routine procedure all suspected material, from which it is desired to isolate *L. monocytogenes*, should be incubated in KT enrichment medium at both 4° and 37° and subsequently plated onto blood nutrient agar, NAB medium and tryptose agar (Difco).

While the described procedure has given good results in our laboratory we wish to stress that other methods may prove more successful in other hands. However, whatever isolation method is used, patience is required in attempts to isolate *L. monocytogenes*. A completely selective medium has not yet been developed.

Notes on Identification of

L. monocytogenes

Even when successfully isolated, *L. monocytogenes* has often been overlooked or been wrongly classified due to certain morphological and cultural resemblances to the enterococci, lactobacilli, corynebacteria and *Erysipelothrix*.

Morphology

L. monocytogenes is a Gram-positive rod. Laboratory cultures (24 h) stained Gram usually show rods $0.5 \times 2-5 \mu$. In older cultures or in rough strains, filamentous structures often appear. However, Gram stains of 24 h cultures of freshly isolated strains show predominantly coccoid forms, $0.5 \times 1 \mu$ in diameter, and can be mistakenly discarded as enterococci.

β -Haemolysis

After 24 h incubation at 37° on blood agar, colonies of *L. monocytogenes* are usually very small, dew-drop like, and sometimes difficult to detect with the naked eye. β -Haemolysis may be scarcely detectable. Suspected cultures should always be incubated for 48 h. Even then the characteristic β -haemolysis is not always pronounced and may not be visible unless the colony is removed from the plate.

Motility

Cultures to be tested for motility should always be incubated in Craigie tubes for three days at room temperature or 22°. At 37° *L. monocytogenes* is

almost never motile and can be mistakenly identified as *Erysipelothrix* or a non-motile coryneform bacterium or lactobacillus

If cultured at the lower temperature all strains of *L. monocytogenes* are motile (Seeliger, 1961). The motility is quite characteristic, quick darting motility is interspersed with tumbling and rotating movements.

Catalase

On nutrient agar the catalase activity of *L. monocytogenes* is often very poor and can be overlooked. The benzidine test (Deibel and Evans, 1960) is almost always negative or scarcely positive. Spectrophotometric studies in our laboratory have shown the cytochrome content of *Listeria* to be very low. This can lead to confusion with lactobacilli or *Erysipelothrix*.

Biochemical tests

Biochemical tests used in the classification of *L. monocytogenes* are described by Seeliger (1961) and Gray and Killinger (1966). We have found the most useful tests to be production of acid from glucose, maltose, salicin, aesculin; no acid production from mannitol, arabinose, raffinose, inulin; no hydrolysis of urea, no reduction of nitrate.

Serological tests

The results of serological tests should always be evaluated in combination with cultural and biochemical tests. Care should be exercised in interpreting the results of agglutination tests because *L. monocytogenes* has a tendency to autoagglutinate. Also misleading results may be obtained because of common serological cross reactions which occur between *L. monocytogenes* and staphylococci, streptococci and coryneform bacteria, probably due to the Rantz antigen (for discussion and excellent description of serological properties, see Seeliger, 1961).

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The Isolation of Non-Pathogenic Yeasts

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The method of sampling the habitat and the composition of the growth medium are two factors of vital importance in any programme for isolating yeasts. Quantitative sampling techniques should be used wherever possible. An estimate can then be made both of the relative importance of each component of the microflora and of the effects of changes in the environment. The use of enrichment techniques or media may be the only means of demonstrating the presence of yeasts forming a minute proportion of a flora, but enumeration is then impossible. Isolation media are normally designed to allow the growth of yeasts alone, but there are many known examples of symbiotic associations between yeasts, moulds and/or acid-tolerant bacteria (Beech and Davenport, 1969). All these organisms should be isolated when examining a new habitat until their role can be determined. The composition of the isolation medium is also important since it can determine the biochemical characteristics of the yeasts (Scarr and Rose, 1966). It should contain either the same sugars as those present in the habitat or glucose alone, since, as shown by Sceda and Yarrow (1966), some cells in a yeast culture can acquire the ability to assimilate and ferment the sugars offered in the medium.

In general terms, a yeast should be isolated using environmental conditions that simulate as closely as possible those found in its natural habitat.

Sampling Methods

With limited isolation programmes a sampling method can be chosen from those described in the literature as being suitable for the particular habitat. But, for an extended programme, it is better to carry out an initial survey using a variety of methods, supplemented by direct microscopic observation, if this is possible. The survey should provide information on the number of organisms removed with the sample, and on any changes in numbers and types of yeast during the preparation of a pipettable slurry.

Such changes can be minimized by chilling the sample during transit and by ensuring that the diluent chosen has no adverse effect on yeast viability (Kamiński, 1958). Samples containing preservatives, inhibitors (Lampen and Weinstock, 1962) or organisms that can inhibit yeasts (Gilliland and Lacey, 1964, 1966; Phaff *et al.*, 1966) will need the addition of neutralizing agents or antibiotics to the initial slurry.

Habitats may be sampled directly or indirectly as indicated below:-

Direct methods

A liquid or slurry can be examined directly under the microscope or after concentration by centrifugation at 2000 r/m for 20 min. The viability of the yeast cells can be determined by mixing the sample with methylene blue (see Appendix); dead cells stain blue while viable cells decolorize the dye. The staining method gives anomalous results with old cells, and a slide culture technique (Gilliland, 1959; Hough, 1960; Report, 1962) must be used instead. Although both methods give some indication of the number and types of yeast forming the major components of the flora, the minor components are readily missed. Furthermore, isolations cannot be made because of the glass cover slip over the preparation. The latter difficulty can be overcome with a single cell isolation technique, using either a standard micro-manipulator or the simple methods of Hansen (1886) or Lindner (1893). A suspension of yeast cells is mixed carefully with melted malt extract gelatin to give a concentration of approximately 100 cells/ml. A drop of this liquid is then spread over the inner surface of a sterile cover slip, previously printed with a numbered grid, and set into a moist chamber sealed with mineral jelly. If the cells are separated sufficiently the chamber can be incubated at 20° to allow growth of viable cells. The micro-colonies formed from single cells can be transferred with sterile needles or rolled strips of filter paper to liquid media. Lindner's method is similar except that drops of the suspension in liquid malt extract are used instead. The methods, described in detail by Jørgensen and Hansen (1948), were devised originally for brewing yeasts, hence the use of malt extract, which should be replaced by the appropriate medium for yeasts from other habitats.

It is not always possible to use the methods described above, either because insufficient yeast cells are present or because they are embedded in an opaque substrate. Under these conditions the presence of yeasts must be demonstrated by the production of visible colonies. The method used will vary according to the nature of the habitat—solid, liquid or gas. Thus the surface of a solid can be examined in several ways, the simplest being the use of an agar sausage (ten Cate, 1965; Beech, 1967), a refinement of the method of Hodges and Wildman (1947). Sausages made of several

agars, including malt, are available commercially (Agaroid, Oxoid Ltd), but sausages of other agar media can be prepared, following the directions of ten Cate (1965). The end of the sausage is removed with a sterile knife and the exposed end pressed on to the surface under examination. A slice is then removed and incubated, impressed side uppermost, in a closed container until visible colonies are produced. Uneven surfaces can be sampled, using a malleable pad (Holt, 1966), velvet pads (Gentles, 1956) or agar held in a piece of gauze (Foster, 1960). Neither these nor any other methods of sampling a surface can be guaranteed to remove all the organisms thereon (Gibbs and Stuttard, 1967). Normally the surface is sampled at random and the experimental error of the counts established statistically. Pressing Sello- or Scotch tape over the surface is another convenient method of examination (Endo, 1966). The impressed tape can be laid on a plug of agar, stained with cotton blue and observed directly through the microscope, or incubated first in a moist chamber: semi-permanent preparations can be made of such impressions (Davenport, 1967). Woodworth and Newgard (1963) have described an applicator for exerting a standard pressure to a known area of Sellotape.

Alternatively, the surface can be washed: Gibbs and Stuttard (1967) consider that self-washing in sterile water is the best method for removing organisms from the human skin. Clark (1965*a, b*) has described a portable apparatus for washing a standard area of any surface. Swabbing the surface with a template, using sterile Johnson's Baby Buds (Johnson and Johnson (Great Britain) Ltd, Slough) and quarter strength Ringer's solution is possible, but introduces the problem of removing organisms quantitatively from the swabs. Alginate wool swabs dissolved in Calgon after use have not overcome these difficulties (Walters, 1967), but can lead to both qualitative and quantitative losses, as shown below for template-defined swabbings of apple tree bark.

Yeasts/cm ²	
Alginate swabs	Cotton wool swabs
350	1008
Species present	
<i>Candida reukaufii</i>	<i>Candida reukaufii</i>
	<i>Rhodotorula glutinis</i>
	<i>Aureobasidium pullulans</i>

Shaking an object with water or liquid media has been used by di Menna (1957) and by Crosse (1959). There is a danger of yeast growth if shaking is prolonged (Beech and Davenport, 1969). Surfaces that are intractable to other methods of sampling can be scraped with a sterile knife. The Castroviejo Keratome (Castroviejo, 1959; Blank *et al.*, 1961) is a sophisti-

cated sampling device for removing sections of horny skin: the strip removed would still require covering with nutrient agar or emulsification and plating on agar media. Sometimes, the sample can be placed on the surface of an agar medium, but it is better embedded in the agar if it is heavily contaminated with fungi; any fungicide incorporated in the medium can then act more efficiently (Egdell, 1967). Alternatively the sample can be rolled over or squashed on to the surface of the agar, but these last three methods are qualitative rather than quantitative.

Membrane filtration is the ideal method for examining the yeast flora of liquids or dilutions of a slurry. It is probably the only method yielding a count of all the organisms present in a fluid. The sample is forced or sucked through a membrane of suitable porosity (normally $1.2\ \mu$ pore diameter), followed by sterile water to dislodge any organisms from the sides of the funnel. The apparatus is dismantled, the membrane removed with sterile forceps and laid on a sterile pad soaked in a rich liquid nutrient medium. Colonies will develop after incubation in a closed glass or aluminium dish. Oils and emulsions, including butter and ice cream, may be membrane filtered after treatment with Triton X-100 (Rohm and Haas Ltd). The technical literature of the manufacturing companies (Millipore (UK) Ltd, Oxoid Ltd and Sartorius Membranfilter GmbH) contain a wealth of detail on techniques and laboratory applications of this method. Alternatively, aqueous samples can be diluted serially and pipetted onto the agar medium, as described for the Presumptive Coliform Count (Report, 1956). The liquid is smeared uniformly over the surface with a curved piece of thin glass rod or Drigalski spatula. The drop count method (Miles and Misra, 1938; Reed and Reed, 1948; Davis and Bell, 1959) may be substituted for very large numbers of samples containing restricted yeast floras.

Probably no method gives a quantitative measure of the yeast flora of gases; there are theoretical objections to almost every method that has been proposed. Four standard methods are used routinely: (i) Bubbling a known volume of air through a measured volume of a liquid medium or quarter strength Ringer's solution. Subsequently the liquid is membrane filtered and the membrane incubated as described previously (Miller, 1963). (ii) The Rotobar/Rotorod sampler described by Asai (1960) and modified by Carter (1961) is a simple, battery-driven device consisting of two rotating vertical arms coated with sticky tape. It has given consistent results over several years in the orchards and ciderhouse of the Research Station. (iii) The Manning slit sampler is a more sophisticated motor-driven device which automatically exposes sections of agar in turn to the organisms carried in on known volumes of air (di Menna, 1955). (iv) Adams (1963, 1964) used the simplest method of all, exposing Petri dishes in the orchard for standard times and culturing yeasts that precipitated on the agar.

Indirect methods

All other types of habitat have to be examined by reducing the sample to a slurry that can be diluted serially prior to plating or membrane filtration. Normally a Waring or similar blender is employed, fitted with an autoclavable jar and lid. It is essential to prevent both increase in numbers due to aeration and frictional heating, as well as any diminution due to the wrong choice of diluent. Clark *et al.* (1954), and Bowen and Beech (1964) found that blending equal weights of sliced apples and chilled sterile water or quarter strength Ringer's solution in a chilled jar for three minutes was sufficient to produce a slurry without serious change in yeast count. Comminution of a one-tenth dilution of the slurry enabled remaining dilutions to be pipetted normally. This method should be used with any heterogeneous sample, e.g. ice-cream containing suspended fruit tissue, smoked cheese with fragments of ham, etc. There is a widespread misconception that fruits only have an external yeast flora and that examination of the interior is unnecessary. But many, if not all fruits, contain yeasts and bacteria in the cores and tissue (Marcus, 1942; Tanner, 1944; Beech, 1958) so that the fruit needs to be comminuted in order to examine the complete flora. Examination of the internal flora of a sample is normally done by first sterilizing the surface, which is then removed with sterile scalpels to reveal the internal structure. Van Uden and Carmo Sousa examined the intestinal flora of swine (1962) and bovines (1957) by alcohol swabbing the selected portion of the digestive tract immediately after evisceration and removing aseptically the contents of selected portions of the internal organs. Methods for examining the internal yeast flora of fruit buds have been described by Davenport (1967).

Enrichment methods

A small concentration of fermenting yeasts can be detected by incubating the sample in a sugary medium and observing the formation of gas bubbles: whole and pulped fruit (Hansen, 1881; Mrak and McClung, 1940; Domercq, 1956), fruit and vegetable slurries (Bowen, 1962) have been examined in this manner. Subsequently the mixture is comminuted and spread on an agar medium for isolation of the colonies. No counts are possible since the yeasts isolated have increased out of all proportion to the numbers present originally. Film yeasts can also be detected in this way and the medium can include concentrations of alcohol ranging from 13–17% v/v for encouraging the growth of wine and sherry yeasts (Scheffer and Mrak, 1950). Ellison and Doran (1961) modified the brewer's classical forcing test to demonstrate the presence of non-fining yeast contaminants in a mass of flocculent brewer's yeast. They claimed commercially significant hazes were produced when

the ratio of contaminant to culture yeast was $1:16 \times 10^6$. Green and Sullivan (1959) proposed the use of synthetic media complete except for one or more of the B group vitamins for demonstrating the presence of minute traces of contaminants in a mass culture of brewer's yeast.

Isolation Media

The isolation medium should be chosen with great care; wherever possible it should be related to the composition of the habitat, fortified if necessary with supplementary nutrients. Thus, apple juice yeast extract agar has proved invaluable for examining the yeast flora of apple orchards, the fruit, juice and ciders (Carr, 1956; Beech, 1957). A medium containing meat extract, whey, hydrolyzed milk and yeast extract was found by Kamiński (1958) to give the highest yeast count for all types of milk tested. Brewer's wort of specific gravity 1.040, or a 5% malt extract or Wickerham's MYPG liquid and solid media (Wickerham, 1951) are used universally in the brewing industry. Naturally, suitable media cannot be made from all substrates. Di Menna (1957) found that one of three agars prepared from soils taken from different sites actually inhibited the growth of all but one yeast. The other two soil agars gave slightly lower counts than a modified Sabouraud agar. Agar media prepared from commercial soft drinks and fruit juices containing such inhibitors as benzoic acid and sulphur dioxide would make unsuitable bases for media, except when searching deliberately for benzoate-resistant yeasts (Ingram, 1959). Sometimes an extract contains a natural inhibitor, e.g. hydroxymethyl furfural, such as found by Lüthi (1958) in apple juice concentrates stored at elevated temperatures. Even unprocessed juices can sometimes contain inhibitors, e.g. *Botrytis cinerea* grown on grapes produces botryticin that is strongly inhibitory to yeasts (Ribéreau-Gayon *et al.*, 1952). Even the juice of sound grapes of the cultivar Chasselas supports much less yeast growth than that of Pinot Noir (Stalder, 1953): it is thought that certain phenolic constituents are responsible for this effect (Masquelier, 1958). Zukerman (1951) considered that the oxidized essential oils of citrus juices could act in a similar manner (see also Ingram, 1956). Therefore, when even a reinforced medium prepared from the habitat is unsatisfactory, recourse must be made to glucose peptone agar. Van Uden and Carmo Sousa (1957), enriched this with yeast extract; Adams (1964) also added beef extract and grape juice.

Media of relatively high pH will allow the growth of non-acid tolerant bacteria. Hence it is normal to reduce the pH to 4.0 by the addition of the calculated amount of hydrochloric or lactic acid between melting and pouring the medium. Should the growth of acid tolerant bacteria still prove excessive, a combination of antibiotics such as the 2 p/m actinomycin and

50 p/m aureomycin used by Beech and Carr (1960) may be necessary. Van Uden and Carmo Sousa (1957) used 50 units/ml penicillin and 100 units/ml streptomycin. Fell *et al.* (1960) and Ross and Morris (1965) used 20 p/m chloramphenicol, 20 p/m streptomycin and 100 p/m chlortetracycline. The lower concentration of streptomycin is preferable since according to Richards and Elliott (1966), this antibiotic can inhibit the growth of some yeast species. When it is necessary to count acid-tolerant bacteria, yeast growth can be prevented by incorporating 10 p/m actidione and 250 p/m 8-hydroxyquinoline in the medium: these two compounds have a complementary action. Mould growth can be inhibited by the addition of the sodium (Lund, 1956) or calcium salts of propionic acid. The actual amount required will depend on the pH of the medium since the fungistatic effect is dependent on the amount of undissociated acid in the medium. At pH 4.8 Bowen (1962) found 0.025% of calcium propionate sufficient to inhibit moulds without noticeable effect on yeast growth. Diphenyl has also been used (Hertz and Levine, 1942; Beech and Carr, 1955) but it is more effective against spores than against hyphal fragments. Rose bengal has been used (Martin, 1950; Adams, 1960 and 1963) for restricting mould growth or for simultaneous counting of yeasts and moulds in a sample. Rose bengal is inhibitory to bacteria and this effect can be reinforced by the addition of 100 p/m kanamycin (Burman, 1965).

Selective media, like the differential media described above, allow the growth of a desired group of organisms without affecting their actual numbers. Selective media permit or demonstrate the growth of yeasts with particular biochemical or physiological properties. Thus Scarr's medium (1959) containing 45% w/v sugar allows only osmophilic yeasts to grow. Such yeasts normally require incubation at 27–30° for optimum growth. Details of methods based on membrane filtration for detecting small numbers of this type of yeast have been given by Devillers (1957) and Scarr (1959). *Debaryomyces* spp. are very halotolerant and will withstand up to 18% NaCl. These yeasts will also utilize nitrite as a sole nitrogen source which Wickerham (1957) has suggested as the basis of a medium for their selective isolation. The medium may be satisfactory where the original microflora has already been reduced by the presence of salt, e.g. cured meats, but for more varied floras such yeasts as *Brettanomyces* spp. would also utilize nitrite (van der Walt, 1963). Lysine (Walters and Thiselton, 1953) has been used routinely for years as the sole nitrogen source in a medium for detecting "wild" yeast contaminants in stocks of brewers' yeast: van der Walt (1962) has suggested the use of ethylamine for the same purpose. Selective media can also be based on single carbon sources such as lactose for isolating *Saccharomyces fragilis* and other yeasts common in milk products; melibiose for yeasts able to ferment raffinose completely and

maltotriose for certain strains of brewing yeasts (Green and Stone, 1952), with Wickerham's synthetic media (1951) supplying the remaining nutrients. Addition of iron in the form of 0.5% ferric ammonium citrate to a biotin-supplemented medium, will cause colonies of *Candida pulcherrima* to assume shades of maroon; these can be distinguished from the pink colonies of carotenoid-forming yeasts by the solubility of the pigment (pulcherrimin) of the first group in methanolic KOH and not in organic solvents (van der Walt, 1952). Acid producing yeasts, *S. acidifaciens*, *Brettanomyces* and *Kloeckera* spp. will demonstrate their presence by clarifying chalk agar.

The compositions of media described above are given in an Appendix at the end of the paper.

Incubation Conditions

It seems obvious that material sampled from the polar ice should be kept frozen in transit, manipulated at 4° and plates of media inoculated from it, incubated at this temperature for several weeks (di Menna, 1960). However, yeasts from other sources may need similar treatment: Peynaud and Domercq (1964) found *C. vanriji* growing in non-sulphited grape juice stored at -1°. Di Menna (1966) put forward a useful policy for choosing incubation temperatures for the psychrophilic yeasts that exist in such habitats. All isolates were incubated at 4° until their temperature optima were known. When this was 15°, subcultures were grown at 5°; when it was 20°, at 15°; and at 20° or room temperature, when the optimum was greater than 20°. According to Nakayama *et al.* (1954), colonies of *Rhodotorula* spp. grown at low temperatures will be yellow, rather than red, due to depression of the red pigments, torularhodin and torulin, and an increase in the yellow, β -carotene. We have had the opposite effect with psychrophilic strains of *Rhodotorula*, one of which, acclimatized to growing at 25°, produced orange-pink colonies that changed to deep coral when held subsequently at 5°.

Dilutions of samples taken in less frigid habitats should be plated in triplicate and incubated at 15°, 25° and 37°. The first for psychrophiles, the second for mesophiles (many of which cannot grow over 30°) and the last for yeasts associated with warm blooded animals. *S. lactis* is one of the exceptions that can exist at 37° or over and not necessarily found in such a habitat. Any yeasts growing at 37° or over may need extra growth factors (Sherman, 1959; Loginova *et al.*, 1962), and in any case should be handled with care since they are potentially pathogenic to humans (Kreger-van Rij, 1961).

Normally plates of media are incubated in the dark but a restricted amount of light is considered to increase the carotenoid production of *Rhodotorula* spp. (Gurinovich *et al.*, 1966). Oppenoorth (1957) produced

evidence that light stimulated spore production in some *Saccharomyces* spp. Ultra violet light is harmful to yeast growth.

The atmosphere over the inoculated plates is not normally controlled during incubation. All yeasts, except *Saccharomycopsis guttulata*, will grow aerobically. Even substituting nitrogen and/or carbon dioxide does not seriously restrict the growth of yeasts normally classed as aerobic or non-fermentative. Carbon dioxide under pressure, e.g. 8 atmospheres at room temperature, will prevent yeast growth.

The period of incubation will vary with the temperature. It can range from 3 to 4 weeks at 4° to 5 to 7 days at 25°, although *Brettanomyces* spp. will need at least 14 days at the latter temperature, unless the medium has been reinforced with biotin and thiamine (van der Walt and van Kerken, 1960).

Isolation Procedure

If the backs of the Petri dishes are not already engraved with a grid, one should be drawn and numbered. Membranes are normally sold printed with a grid. These grids enable the entire surface of the plate or membrane to be examined systematically under a plate microscope ($\times 10$ magnification). The characteristics of each colony type are listed under the headings of colour, surface topography, degree of glossiness, texture, cross-section, size and appearance of the periphery. There are appropriate illustrations in Salle (1961) with standard names for colonial features. A wet smear of each colony type is examined microscopically and the appearance of the cells also listed, together with the code number of the colony: this number should be assigned to the isolate made from the remainder of the colony. Counts should be recorded of each colony type, together with the total yeast count, size of sample plated or filtered, the degree of dilution, medium used, incubation conditions, date of sampling, sample treatment and an accurate description of the habitat from which the sample was removed. Often the yeast will exhibit macro- or micro-characteristics which may disappear on sub-culturing, e.g. the ability to form asco- or ballisto-spores. Unless these characteristics are recorded accurately it may be impossible subsequently to assign the yeast to its correct genus.

Normally the isolates from each plate are streaked thrice to test for purity, although yeasts forming "rough" colonies may need prolonged re-streaking before purity can be established. The pure cultures must then be stored on the same type of medium to avoid colonial changes induced by alterations in the composition of the medium.

Anyone unfamiliar with the appearance of yeast colonies should consult Etchells *et al.* (1953), de Becze (1959b) and Richards (1967). The appearance

of yeast cells has been described by Lodder and Kreger-van Rij (1952), de Becze (1959a) and Phaff *et al.* (1966).

Storage of Yeast Cultures

The purified culture needs to be stored under conditions that leave its morphological, physiological and biochemical properties unchanged. Thus, it can be stored on the same medium as was used for its isolation or on Sabouraud's agar with glucose as the sole sugar. Brewing yeasts can be stored on malt extract or wort-agar or MYPG agar. Osmophilic yeasts need to be stored on Scarr's medium (1951), wine yeasts on media containing 12% ethanol (Scheffer and Mrak, 1950) or in sterile-filtered dry white wine. Acid-forming yeasts, in particular, *Brettanomyces* and *Kloeckera* spp., require chalk agar and transfer at quarterly intervals. The former group would also benefit by the addition of 10 mg thiamine/litre: similarly fat-splitting yeasts should have added oleic, myristic or palmitic acids. The temperature of storage is usually 0–5°, with sub-cultures prepared annually. Dehydration of the medium and mould growth can be prevented by covering the inoculated agar with sterile mineral oil (Henry, 1947), using screw-capped bottles and self-shrinking plastic covers (Beech, 1957). In spite of the advantages of these methods, the labour of sub-culturing and media preparation is causing more and more culture collections to be stored as freeze-dried specimens. The centrifuged cultures are suspended in sterile horse or bovine serum albumin or, at Long Ashton, in Carr's modification of Stamp's medium (1947), see Appendix. Such dried cultures are stored at 0° and sub-cultured every two years. Lyophilization does tend to induce changes in the vitamin requirements of yeasts and the formation of respiration deficient cells (Wynants, 1962) but this can be guarded against when selecting colonies after growing the revived culture on solid media. There is no evidence to suggest that lyophilization changes important characteristics such as growth curves, flocculation, sporulation, fermentation or assimilation reactions (*cf.* Wickerham and Flickinger, 1946; Kirsop, 1955; Haynes *et al.*, 1955; Brechot *et al.*, 1958; Brady, 1960; Martin, 1964).

Testing Yeast Cultures for Purity

At intervals stored yeast cultures should be checked for the presence of contaminants.

Bacteria can be detected by streaking the culture on a suitable medium containing the yeast inhibitors of Beech and Carr (1955, 1960) or, for yeasts sensitive to actidione, the media proposed by Williamson (1959). The presence of mould spores can be detected more readily by incorporating both yeast and bacterial inhibitors in the medium (pp. 76–77). Wild yeasts

in cultures of *S. cerevisiae* and *S. carlsbergensis* can be detected by streaking on plates of lysine agar (Walters and Thiselton, 1953). Similarly petite, or respiration-deficient yeasts react differently from normal yeasts on Nagai's medium (1963, 1965): Kleyn and Vacano (1963) incorporated triphenyl-tetrazolium chloride in their medium for this purpose (*cf.* Sherwood and Hines, 1959).

At least 50 single cell isolates should be prepared (p. 72) from the stock culture once the gross contamination has been removed: giant colonies are then prepared from these isolates (Richards, 1967). If they are identical in appearance, a further 50 colonies should be prepared and subjected to the tests which characterized the original. If the colonies have the same appearance and reactions, then a new stock culture should be prepared from one of them. Industrial yeasts should also be examined for those characters that make them suitable for the industry they are meant to serve. Gilliland (1951), Hough (1957) and Stevens (1966) have described suitable tests for brewing yeasts; others exist for baking, wine, food and distillery yeasts.

Appendix

Many of the media described below are available commercially in dehydrated form from either Oxoid Ltd, Southwark Bridge Road, London, S.E.1, or from Difco Laboratories, Baird and Tatlock Ltd, Freshwater Road, Chadwell Heath, Essex.

Percentages quoted below are % w/v, unless otherwise stated.

Apple juice yeast extract agar

Deep-frozen, depectinized apple juice is diluted to specific gravity 1.045 and fortified with 1% Difco Yeast extract and 1 mg thiamine/litre. The pH is adjusted to pH 4.8 with sodium hydroxide pellets and the mixture filtered through clarifying membranes. The mixture is dispensed and sterilized by autoclaving at 121° for 15 min. The medium can be solidified with 3-4% Ion agar, Oxoid.

Apples low in tannin should be chosen, such as Bramley's Seedling or Cox's Orange Pippin. The apples should be sliced, heated to 35° and stirred with the enzyme Rohament P and a good commercial depectinizing enzyme. After 2 h the juice can be obtained by squeezing the pulp through muslin. Juice can be prepared from other fruits in the same manner.

Carr's suspending medium for lyophilizing yeasts

Gelatin 1%, Difco yeast extract 1%, glucose 0.5%, ascorbic acid 0.25%, pH adjusted to 5.5 and sterilized by steaming for 30 min on three successive days.

Chalk agar

Glucose 5%, Difco Yeast Extract 2%, reprecipitated calcium carbonate 0.5%, and agar 2% in tap water. The dispersed ingredients are dispensed and autoclaved at 115° for 15 min. To pour plates, the medium is melted, cooled to 50°, mixed to disperse the chalk and poured into the sterile plates which are refrigerated immediately to solidify the agar.

*Lysine agar**Medium A*

0.1 g Boric acid
0.04 g Zinc sulphate. $7\text{H}_2\text{O}$
0.02 g Ammonium molybdate
0.04 g Manganese sulphate. $4\text{H}_2\text{O}$
0.25 g Ferrous sulphate. $7\text{H}_2\text{O}$
1 litre Distilled water

Medium B

50 g Glucose
2.0 g Potassium dihydrogen phosphate
1.0 g Magnesium sulphate. $7\text{H}_2\text{O}$
0.2 g Calcium chloride (fused)
0.1 g Sodium chloride
0.002 g Adenine
0.001 g DL-Methionine
0.001 g L-Histidine
0.001 g DL-Tryptophan
1.0 ml Medium A
12.0 ml Potassium lactate (50% w/w)
1 litre Distilled water
20 g Agar
pH 5.0–5.2 with lactic acid

Medium C

10 g Lysine
1 litre Distilled water

Medium D

2.0 g Inositol
0.2 g Calcium pantothenate
0.04 g Thiamine
0.04 g Pyridoxin
0.02 g p-Aminobenzoic acid
0.04 g Nicotinic acid
0.02 g Riboflavin
0.0002 g Biotin
0.0001 g Folic acid
1 litre Distilled water

Media B, C and D are sterilized separately by steaming for 30 min on three successive days, then mixed in the following proportions while the liquids are at 45 to 50°:

Medium B 89 parts
Medium C 10 parts
Medium D 1 part

Malt extract

Microbiologists in the brewing industry often use the extract prepared from malted barley. This is known as wort and is normally standardized to a

standard soluble solids content, e.g. 10° Balling or specific gravity 1.040. Otherwise a malt extract liquid or agar is prepared from the concentrated extract or the spray dried product. A stable clear medium can be prepared as follows:

200 g spray-dried malt is dissolved in 1 litre tap water, adjusted to pH 5.4 and autoclaved at 121° for 15 min. The liquid is then boiled for 1 h, any evaporation losses being made up with distilled water. Chilling and refrigeration for at least 1 h follows before clarifying the solution with kieselguhr. The solution is diluted to specific gravity 1.060 for a liquid medium and to 1.040 and solidified with 4% agar for a solid medium. Sterilization is by steaming for 30 min on three successive days. 10% gelatin can be used instead of the agar for malt extract gelatin.

Methylene blue

2 g of sodium citrate dihydrate is added to 10 ml of aqueous 0.1% methylene blue solution. The mixture is made up to 100 ml with distilled water.

MYPG

Spray dried malt extract 0.3%, Difco yeast extract 0.3%, Eu-peptone (Allen and Hanbury) 0.5%, glucose 1% in distilled water. Solid medium prepared by the addition of agar 3%. Both liquid and solid media are sterilized by autoclaving at 121° for 15 min.

Osmophilic agar

Difco wort agar is dissolved in a 45° Brix syrup containing 35 parts of sucrose and 10 parts of glucose. It is sterilized by autoclaving at 108° for 20 min and may be remelted 3 times without serious colour formation.

Sabouraud agar

1% peptone, 4% glucose and 1.5% agar, sterilized at 121° for 15 min. The pH is approximately 5.2 but is often adjusted to 4.0 by the addition of lactic or hydrochloric acid between melting and pouring the medium.

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The Isolation of Myxomycetes

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The isolation of a micro-organism implies to most microbiologists the achievement of sustained culture in the absence of other micro-organisms (i.e. *pure* or *axenic* culture), usually on a soluble medium, either liquid or solidified with agar. Judged by these criteria, very few Myxomycetes have been isolated; the types of culture listed below represent steps in achieving the ideal of pure culture on soluble media.

1. *Crude culture*. Many Myxomycetes can be grown in the laboratory in crude culture on such substrates as oatflakes sprinkled onto plain agar. No attempt is made to eliminate other micro-organisms, apart from any which may hinder the growth of the Myxomycete. Most research on Myxomycetes has so far been carried out with such cultures, which are, moreover, essential as a source of inoculum for attempting pure culture.

2. *Two-membered culture*. Some Myxomycetes can be freed from contaminating micro-organisms, and then successfully cultured in the presence of a second known microbial species, commonly a bacterium or yeast.

3. *Pure culture on particulate substrates*. A few Myxomycetes can readily be grown in pure culture on particulate substrates, such as sterile oatflakes sprinkled onto plain agar.

4. *Pure culture on soluble media*. As yet very few species have been grown in pure culture on soluble media, and none throughout their life-cycle. Such culture permits the application to Myxomycetes of all the usual techniques of microbiology and biochemistry, and is well worth achieving.

The remarkable Myxomycete life-cycle will now be described, as a preliminary to an account of the methods employed in achieving crude culture and attempting pure culture of these organisms.

Life Cycle

The most striking feature of the Myxomycete life-cycle (Fig. 1) is the plasmodium. This is a multinucleate mass of protoplasm of irregular shape, sometimes covering an area of many square centimetres, and capable of

migration when conditions become unfavourable. In many species the plasmodium has a conspicuous network of "veins" (Fig. 2), channels in which rapid protoplasmic streaming occurs, with reversals in the direction of flow taking place about once per minute. In some species it has been established that the nuclei in a plasmodium divide synchronously.

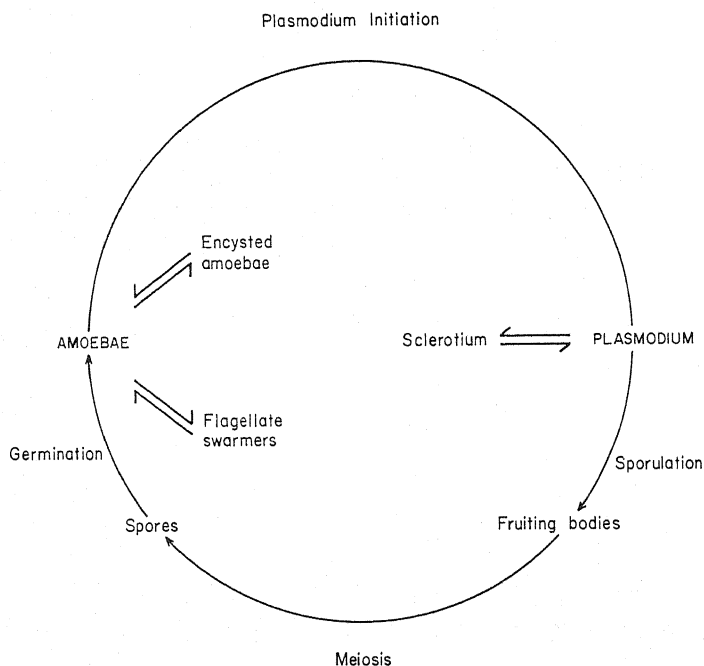


FIG. 1. Life cycle of a Myxomycete.

Plasmodia may be maintained in the active state indefinitely by frequent subculture. Otherwise, nutrient exhaustion, which in a Petri dish cannot be evaded for very long by migration, leads to the production of either a resting phase (the sclerotium) or fruiting-bodies (Fig. 3) containing spores (sporulation). The form and structure of the fruiting bodies, which are often complex, is the main criterion used in the classification of Myxomycetes. In many species light is required for sporulation, but sclerotium formation can proceed in darkness.

Spores dispersed from fruiting bodies germinate to give rise to amoebae which, like plasmodia, may be propagated indefinitely. The amoebae may become swimmers (i.e. elongate and develop flagella) in the presence of water, and will encyst when food is exhausted. Encysted amoebae survive for long periods. Plasmodium formation, in some species, is initiated by a

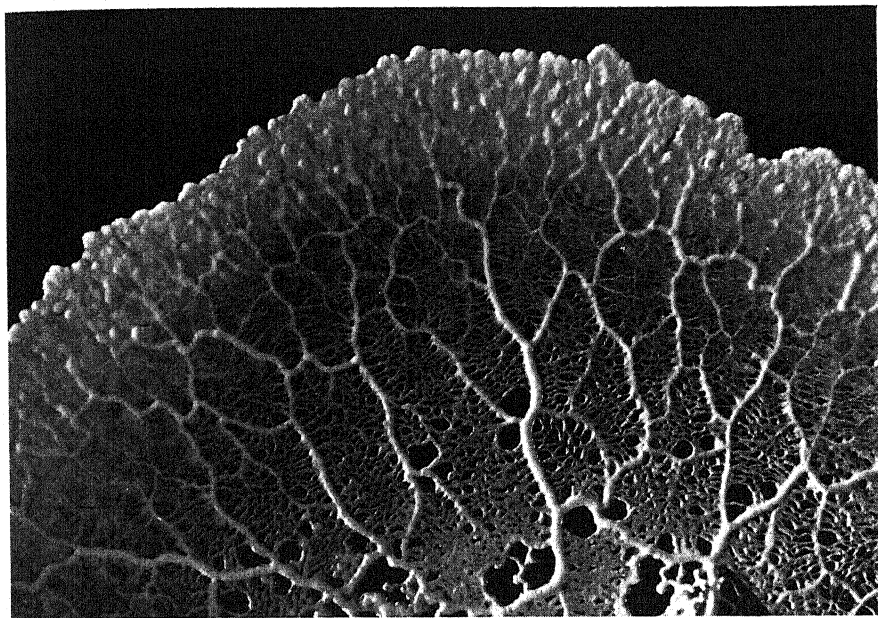


FIG. 2. Plasmodium of *Physarum polycephalum* in pure culture on an agar medium. Note "veins" ($\times 4.5$).

mating reaction between two amoebae genetically different at a mating-type locus, but in other species mating can take place between amoebae of the same clone, and in some it is possible that mating is not necessary for plasmodium initiation.

Myxomycete amoebae are haploid, meiosis having occurred during spore formation. Plasmodia are generally thought to be diploid, with nuclear fusion accompanying cell fusion at mating, but in some species it is possible that plasmodia are dikaryotic, with nuclear fusion deferred until shortly before meiosis, at the time of sporulation.* More detailed accounts of the Myxomycete life cycle are provided by Alexopoulos (1962, 1963, 1966).

Collection and Crude Culture

Collection

Myxomycetes are common, especially in damp woodland in the autumn on decaying logs, fallen leaves and dead vegetation, where they feed on other micro-organisms. Fruiting bodies are particularly abundant but readily

* Recently Kerr (1968) has shown that in *Didymium nigripes* the amoeboid and plasmodial phases do not differ in ploidy and hence that in this species nuclear fusion and meiosis do not occur.

overlooked, except by those who know what to look for and where to look; the author recalls a foray near London in which an expert found 13 Myxomycete species before lunch whereas others present failed to find any. It is advisable, therefore, to make a first collecting trip in the company of a naturalist or mycologist familiar with the Myxomycetes. Plasmodia are less frequently found than fruiting bodies but are more conspicuous.



FIG. 3. Fruiting bodies of *Didymium melanospermum* on dead vegetation ($\times 3.35$).
Photograph by Mr Bruce Ing.

Specimens are collected along with the substrate to which they adhere, and a sharp penknife is useful for cutting away portions of wood bearing plasmodia or fruit bodies. Transport to the laboratory presents problems, as fruit bodies are fragile, and plasmodia easily killed by crushing, and perhaps the best method is to use a metal box with hinged lid, lined in the bottom with cork, and to fix specimens on to the cork with bead-headed pins as soon as collected (Mr Bruce Ing, personal communication). On arrival in the laboratory, specimens with fruiting bodies may be identified with the aid of the monograph by Lister (1925) and the more recent taxonomic literature cited by Alexopoulos (1963); plasmodia, however, can only be identified if they subsequently sporulate. Fortunately, permanent preparations of fruiting bodies can readily be made (Lister, 1925) and sent to an expert for identification should this be necessary.

Crude culture of plasmodia

Decaying wood and leaves probably bear mites, so it is best to carry out the first steps in isolation well away from cultures which might become infected,

and to transfer crude cultures of Myxomycetes to the incubator only when convinced that mites are no longer present. Wood or leaves carrying plasmodia can be placed on sterile agar in a Petri dish; the plasmodium soon migrates away from its original substratum. Spores from crushed fruiting bodies can be placed on an agar medium (e.g. corn meal agar) which permits some but not excessive bacterial growth. Germination to give amoebae soon takes place and, if the investigator is fortunate, plasmodia are subsequently produced. Myxomycetes may be sub-cultured by cutting out part of a plasmodium and the underlying agar with a sterile scalpel or similar tool, as cutting does little harm to plasmodia although crushing is highly injurious. When cultures are transferred to the incubator, 22° or 24° is usually the maximum that can safely be employed, most Myxomycetes being unable to live at much higher temperatures.

Sustained crude culture requires the elimination of harmful contaminants that might overwhelm the plasmodium, and the provision of a suitable nutrient substrate. The former is usually not difficult, as the plasmodia migrate rapidly on plain agar which enables them to escape from filamentous fungi, the most troublesome common contaminants. Provision of a suitable substrate is more of a problem as too abundant nutrients (possibly themselves directly harmful) lead to excessive development of bacteria which in turn poison the plasmodium with their metabolic products. For the crude culture of many Myxomycetes, a few sterile rolled oats or porridge oats sprinkled on to plain agar are satisfactory. Under these conditions the plasmodium is probably nourished partly by the oats and partly by a limited growth of the bacteria present. Some Myxomycetes which will not grow on this medium will thrive on live yeast streaked thickly on to plain agar. Various yeasts may be tested to find which is most effective: *Saccharomyces cerevisiae* is often satisfactory.

Pure Culture and Two-membered Culture

The plasmodia of some Myxomycetes will grow well in two-membered culture, as shown by the work of Cohen (1939, 1941) and Sobels and Cohen (1953), but most workers wishing to advance beyond the crude culture of plasmodia have favoured pure culture, two-membered culture being merely a step in the elimination of contaminants. However, only a few Myxomycete amoebae have been grown in pure culture, such as *Didymium nigripes* on formalin-killed (Kerr, 1963) or heat-killed (Schuster, 1964) bacteria, and *Badhamia obovata* (*B. curtisii*) on a soluble medium (Ross, 1964), two-membered culture being a more practicable procedure. Hence we are concerned mainly with establishing pure cultures of plasmodia and two-membered cultures of amoebae.

Elimination of contaminants from plasmodia

A migrating plasmodium readily escapes from colonies of filamentous fungi, but bacteria are not so easily eliminated. Migration across several dishes of plain agar may be necessary to eradicate all contaminants, and before this is achieved the plasmodium may have died or become a sclerotium through starvation. In order to avoid this, Cohen (1939) introduced an "enrichment"

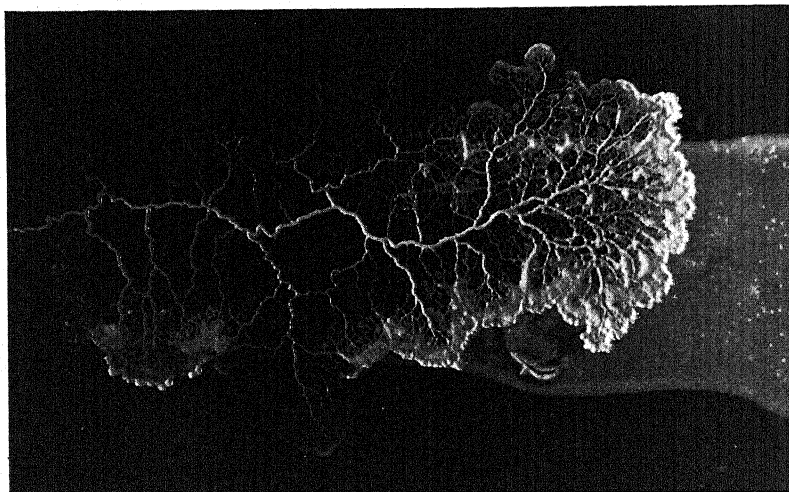


FIG. 4. Migration of plasmodium of *Physarum polycephalum* along a streak of washed yeast (*Saccharomyces cerevisiae*) on plain agar ($\times 3.5$).

procedure—essentially achieving pure culture *via* a brief phase of two-membered culture with a readily eliminated species. A plasmodium is permitted to migrate along a streak of yeast on plain agar (Fig. 4). This, repeated if necessary, will effectively remove all contaminants except the yeast, yet the plasmodium will remain well nourished. Migration on plain agar will then free the plasmodium from yeast, the cells of which, being fewer and larger than those of bacteria, are sooner eliminated. Alternatively, migration along streaks of *Escherichia coli* may be employed, followed by its elimination by means of migration on plain agar, acidified to pH 4.6–5.0 (a degree of acidity favourable for many Myxomycetes) with, if necessary, the addition of 1 g/litre streptomycin. A second antibiotic tolerated well by Myxomycetes is penicillin, so, instead of *E. coli*, penicillin-sensitive bacteria could be used for "enrichment".

Plasmodia are then transferred to the agar media chosen for pure culture and as soon as vigorous growth has occurred, tests for the presence of

contaminating micro-organisms are carried out. Such tests are best made both with the medium to be used for pure culture of plasmodia, and media suitable for detecting the most likely contaminant—the species used in the enrichment procedure. The tests are carried out in test tubes half-filled with liquid medium, large pieces of plasmodium and adhering agar being placed at the bottom. Plasmodia inoculated into unshaken liquid medium do not grow hence the test-tubes may be subjected to prolonged incubation at the temperature employed for Myxomycete culture to give contaminants ample time to develop.

Pure culture of plasmodia

Relatively few Myxomycetes can be maintained routinely in pure culture, so suggestions as to media suitable for attempting such cultures are necessarily tentative. Some species will grow well on a plain agar medium on which a few sterile oat flakes have been sprinkled; some workers, however, prefer to incorporate the oatflakes into the agar. Autoclaved yeast spread on plain agar or a buffered, non-nutrient agar has been successfully employed by some workers.

Pure culture of plasmodia on completely soluble media, in both agar and shaken liquid culture, has been achieved with *Physarum polycephalum* (Daniel and Rusch, 1961); such media contain glucose, peptone, mineral salts, biotin, thiamin and haematin (Daniel and Baldwin, 1964). The need for haem or haemoproteins was clearly demonstrated (Daniel *et al.*, 1962), so the possibility that other Myxomycetes have this requirement should be considered in devising media for attempted pure culture. Sporulation of *P. polycephalum* will occur both on oatflakes and on soluble media after growth is complete, exposure to light being necessary to initiate the process. Ross (1964) has succeeded in growing plasmodia of two other species on a medium based on that of Daniel and Rusch (1961).

Two-membered culture of amoebae

The amoebae of *P. polycephalum* are readily grown (Dee, 1962, 1966) in two-membered culture with *E. coli*, dilute liver extract (0.05%) agar being inoculated with a suspension of encysted amoebae and *E. coli*. On this dilute medium, the bacterium grows sufficiently to provide nourishment for the amoebae without overwhelming them. Cultures are established in the first instance by germinating spores produced by plasmodia either in pure culture, or in two-membered culture with *E. coli*, thus avoiding the problem of eliminating contaminants. The possibility of plasmodia arising in amoeba cultures can be avoided by cloning the amoebae, as *P. polycephalum* is heterothallic. Essentially, this is achieved by diluting the suspension of

spores or encysted amoebae employed, so that each spore or encysted amoeba that germinates gives rise to a single colony. Such colonies are visible to the naked eye as clear circular areas in the opalescent film of *E. coli* at the agar surface, due to the digestion of the bacteria by the amoebae. Amoebae from such colonies may be used to establish clones.

Two-membered cultures of Myxomycete amoebae may be established with other bacteria, such as *Aerobacter aerogenes*, employed by Kerr and Sussman (1958) for culturing *Didymium nigripes*. This Myxomycete is homothallic so plasmodium initiation cannot be avoided by cloning; it was however prevented by the incorporation of 2% glucose or 0.2% brucine (a compound very toxic to man) in the medium. Many workers have established what are effectively two-membered cultures of amoebae from crushed fruiting bodies collected in nature. The elimination of contaminant bacteria from such cultures does not seem to have been discussed; possibly contaminants disappear in the course of repeated sub-culture as a consequence of vigorous competition from the chosen bacterial species, or perhaps the possibility of a few contaminant bacteria being present has not been considered troublesome. The elimination of contaminant bacteria from two-membered cultures of the amoeboid cellular slime moulds (Acrasiales) has been described by Raper (1951); his methods might be applicable to Myxomycete amoebae.

Discussion

The first convincing account of two-membered and pure culture of Myxomycete plasmodia was that of Cohen (1939); this paper remains valuable, as does the review by Sobels and Cohen (1953). References for the crude culture from "spore to spore" of 28 species are listed by Alexopoulos (1963), and Daniel and Baldwin (1964) describe methods for the pure culture of the most intensively studied species, *Physarum polycephalum*. Studies on Myxomycetes are, however, at an early stage and the literature is often contradictory. Hence procedures for the isolation of Myxomycetes described here and elsewhere must be regarded as tentative—reliable routines have yet to emerge.

Myxomycetes are sometimes described as non-cellular slime moulds, and the Acrasiales as cellular slime moulds. The use of the term slime mould for both groups is unfortunate, as it has resulted in confusion, even among microbiologists, between two groups of organisms that have little in common except in having a complex life-cycle that includes an amoeboid phase. Far more work has been done on the isolation and two-membered culture of cellular slime mould amoebae than on those of Myxomycetes; valuable accounts of methods are provided by Bonner (1967), Cavender and Raper (1965) and Raper (1951).

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Isolation of *Phytophthora* and *Pythium*

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Though fungi of these two genera may be isolated by the conventional mycological methods such as picking up likely hyphae or plating out bits of infected tissue, a more sure method is one that relies on the fact that the vegetative spores (zoospores) are motile in water. Also, after having settled down, they are able to grow saprophytically on almost any kind of medium or substratum.

These fungi exist mostly in soil and cause root rots and collar rots, though a few can attack leaves and fruits or even tree trunks; they cause well known diseases like potato blight (*Phytophthora infestans*), red stele root disease of strawberry (*P. fragariae*), leather rot of strawberry fruits (*P. cactorum*), stripe canker of cinnamon trunks (*P. cinnamomi*), stem burn of tobacco (*Pythium deliense*), soft rot of melon and squash (*Pythium* spp.), etc., and also damping-off of seedlings.

Isolation Techniques

From water and soil

They are often present in water supplies, particularly irrigation water, and can be detected in water and in soil and isolated therefrom by the "bait" method. This consists in "baiting" the water or soil *in situ*, or samples of either taken to the laboratory, or mixed soil and water, with plant organs on which the fungi will grow readily, e.g. seeds, seedlings, succulent fruits, leaves or leaf pieces (see "suitable baits" below). This "baiting" method has been practised for many years and its various applications are detailed by Sparrow (1960); Fuller and Poyton (1964) have described a continuous centrifuge method for concentrating zoospores in large volumes of natural water.

The "bait" (or several preferably) is placed in the substratum for a suitable period, determined by withdrawing samples at consecutive intervals of time. It is then well washed and transferred with fresh "bait" to fresh water (either glass distilled sterile or, more satisfactorily, a mixture of a third pond: two-thirds distilled, autoclaved 121° for 15 min). Tap water often

contains substances toxic to these fungi (Goode, 1956; Emerson, 1958). In this way, usually, a monoculture is obtained which can be transferred to agar and cleansed by the conventional purification methods (see "purification" below). Another way with suspected soil is to pack a small quantity in a cavity in an apple which is sealed; later the fungus can be recovered from the flesh some distance away (Campbell, 1949). Almost pure cultures of *Phytophthora* spp. have been obtained by burying oranges in citrus orchard soils suspected of harbouring these diseases; avocados in soils with avocado root rot (Zentmyer *et al.*, 1960), pineapple leaves near parent diseased plants (Anderson, 1951; Klemmer and Nakano, 1962), sisal leaves in plantations with zebra leaf spot (Wienk and Peregrine, 1965), fruits in suspected irrigation water (McIntosh, 1966), lupin seedlings in pine root rot soil (Chee and Newhook, 1965), and *Pythium* spp. by brassica seedlings in market garden soils (Barton, 1958).

From roots

For isolation from diseased root systems, the roots are first well washed, in running water over night if much bacterial contamination is anticipated, and then the whole system (if small) or representative parts (if large) are submerged in shallow pond water mixture in 4 in. (10 cm) Petri dishes. Surface sterilization is not recommended unless there is very heavy contamination. After 24 h and on successive days the dishes are searched under a binocular microscope for hyphae and sporangia of *Phytophthora* or *Pythium* which grows out readily into the water. Pieces of root bearing these are picked out, put in fresh water, "baited", usually with small pieces of grass blade (boiled for 10 min, Emerson, 1958) or with small seeds (cold sterilized in propylene oxide, Hansen and Snyder, 1947). If the root pieces appear to contain only one readily growing species, small sections (2 or 3 mm) plated out will usually yield monocultures. Otherwise the "bait" can be renewed and plated when it appears to bear a single strain.

From aerial parts

For aerial parts—collar, stem, leaves or fruits—after thoroughly washing, thin slices are cut at what appears to be the advancing edge of the rot, usually marked by a discoloration. These are floated in shallow water and treated in a similar manner to the root pieces.

These methods can also be used for separating mixed cultures, for example, a species with swimming spores can be separated from one with non-motile spores by picking up the zoospores on "bait", or two species with motile spores can be separated if they produce, or can be induced to produce (probably by growing at different temperatures) sporangia and zoospores at different times.

Purification

Purification of cultures may be achieved by growing the primary material on plates of plain distilled water agar with a dry surface (poured the previous day and left to dry). Japanese agar is recommended; specially purified agars (e.g. Difco, and Oxoid Ionagar No. 2) inhibit the growth of these fungi. Usually *Pythium* and *Phytophthora* spp. will outgrow bacteria on this medium, and as the hyphae are fairly widely spaced, those apart from other contaminants can be cut out and replated. Where bacterial contamination is heavy, especially in warm or hot climates, it may be necessary to use an antibiotic, e.g. pimarinin (Eckert and Tsao, 1960) or to grow at a reduced temperature, 10°–15° in a refrigerator.

Suitable "baits"

Seeds or seedlings: hemp (*Cannabis*), stock (*Matthiola*), *Brassica*, poppy (*Papaver*), antirrhinum, calceolaria, parsley, lupin.

Leaves: grasses, pineapple, sisal.

Fruits: hard tomatoes, small apples or pears, rose hips, crataegus, pyracantha, citrus, avocado.

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Isolation of Pathogenic Fungi from Waterfowl

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Numerous species of fungi occur in or on birds (Beer, 1963; Pugh, 1965) but the majority are fortuitous contaminants. Those that cause disease or structural alterations are of special interest. In waterfowl the most important fungus is *Aspergillus fumigatus* Fresenius. *Candida albicans* Berkhout occurs much less frequently, while *Mucor pusillus* Lindt and *Cladosporium herbarum* Link ex Fries seldom cause disease although common in the environment.

Since so many different organisms, including yeasts and bacteria, are normally present on the avian host, cultural conditions must be highly selective to reduce overgrowth by unwanted species. This can be achieved to a varying extent by the use of a mineral salts-sugar medium (Czapek Dox) at a low pH and an incubation temperature well above or below the usual 25° used for most fungi. Some additional degree of selectivity can be provided by taking inocula from specific tissues.

Although this paper is primarily concerned with the isolation of certain fungi from the avian host, the techniques are readily adaptable to the isolation of the fungi from other environments.

Aspergillus fumigatus

In birds this species causes the severe and often lethal respiratory disease of aspergillosis. It is particularly common in waterfowl; in ducklings it is normally a primary disease but in older birds it is a secondary condition. The dry conidia are inhaled from the atmosphere or during feeding when the conidia are dispersed into the air. The litter layer in grasslands, grain, straw and hay are common sources, especially when decomposing at high temperatures.

A. fumigatus is not a fastidious organism and grows moderately rapidly on Czapek Dox agar. A modified form is used with an antibiotic and a low pH to reduce bacterial growth. The medium contains:

NaNO ₃	3.0 g
KH ₂ PO ₄	1.0 g
KCl	0.5 g



FIG. 1. Caustic-potash ink digest of aspergillosis lesion from a bird's lung. The hyphae are septate, narrow and often highly branched.

MgSO ₄ ·7H ₂ O	0.5 g
FeSO ₄ ·7H ₂ O	10 mg
Sucrose	30 g
Chloramphenicol	10 mg
Agar	15 g
Tap water	1 l
pH, adjust with HCl to 4	

Four ml amounts are pipetted into 7 ml bijou McCartney bottles, sterilized at 115° for 10 min and allowed to set as stabs. The same medium can be used in plates.

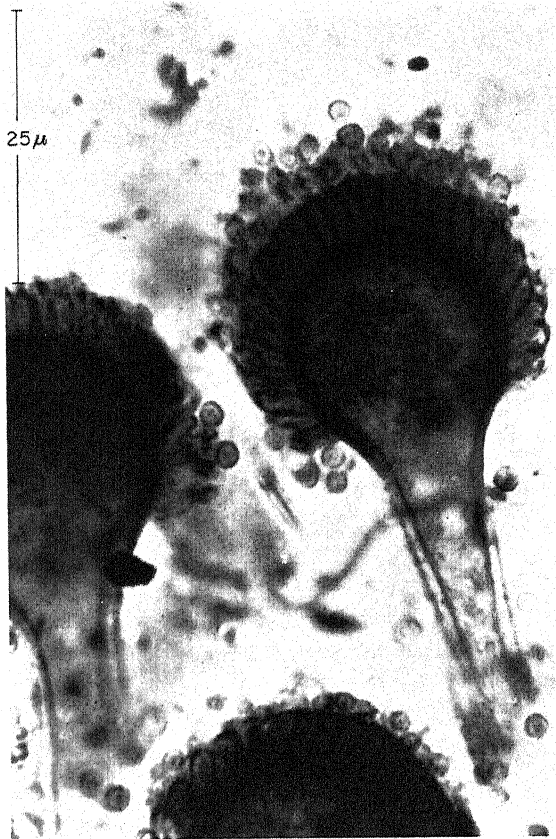


FIG. 2. Conidial head of *Aspergillus fumigatus* with a single row of sterigmata and small conidia.

It is always helpful to have some idea of what type of fungus is present in the tissue. Suspected lesions are digested in 20% potassium hydroxide on a slide heated over a 60 watt light bulb contained in a simple box-shaped housing. Mycelium can often be seen without further procedures under the microscope, but a drop of permanent blue-black ink (Quink) will stain any hyphae in the digest deep blue against a red or pale blue background. Depending on the type of lesion *A. fumigatus* has narrow ($3\ \mu$) hyphae with short or long branches (Fig. 1).

After checking the hyphal type, a portion of a nodule or plaque from the respiratory tract is transferred to an agar stab and pushed below the surface of the agar to ensure rapid action of the selective conditions. The

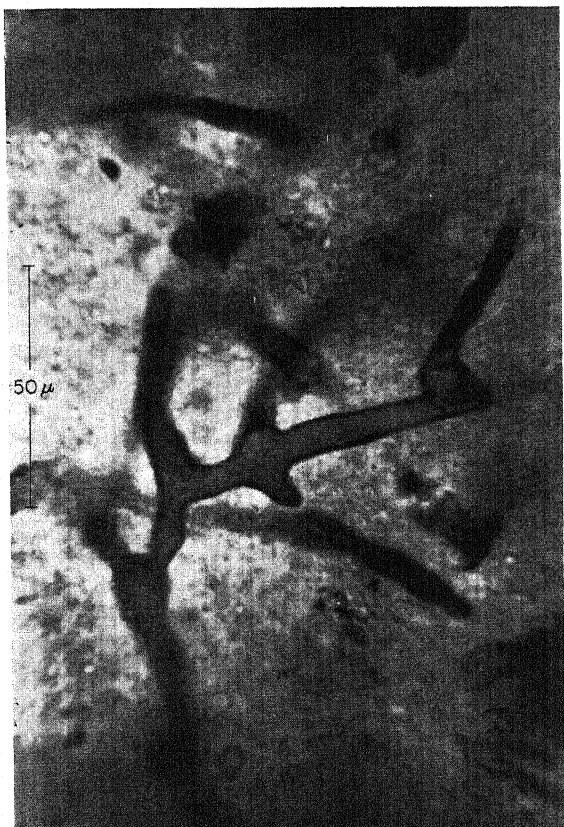


FIG. 3. Caustic-potash ink digest of lesion from respiratory phycornycosis in a bird's lung. The hyphae are relatively wide, coenocytic and in places swollen.

culture is incubated for 2–3 days, with the bottle cap loose, at the optimum growth temperature of 40°, a temperature at which many fungi are unable to grow. *A. fumigatus*, which grows as a dull green, velvety colony, is readily identified by the conidial structures (Raper and Fennell, 1965) (Fig. 2).

Plate or stab cultures can be used to isolate *A. fumigatus* from the environment. Suspensions, diluted if necessary, of soil and organic debris can be inoculated on to the medium, or plates can be exposed to the air for crude assessments of conidial content. Often pure cultures are obtained on incubation but phycornycetes can sometimes be troublesome, especially when rotting debris is used for the inoculum.

This organism must be handled with care as it can cause aspergillosis in

man. The dry conidia are very easily dispersed into the air from sporulating lesions and cultures, and it is advisable to handle the material in some sort of ventilated hood if regular work is to be done with *A. fumigatus*.

Mucor pusillus

M. pusillus is the cause of a rare respiratory phycomycosis of waterfowl, the lesions of which are not unlike those of aspergillosis (Beer, unpublished data). Caustic potash-ink digests show wide (5–10 μ) and sometimes swollen coenocytic hyphae (Fig. 3).

This organism is isolated in the same way as *A. fumigatus* but growth is improved by the addition of 0.1% glucose to the modified Czapek Dox agar. It grows rapidly at 40° as a brownish grey, 1–2 mm high, sporulating colony (Gilman, 1957). If both phycomycosis and aspergillosis are suspected, care must be taken when selecting the lesion for the inoculum. If sporulation has occurred the plaques of the former will be grey and the latter green. In the lung the nodules are respectively soft and hard. Alternatively, lesions can be dispersed in sterile saline, diluted if necessary, and plated out.

M. pusillus is not often found in the air but it is frequently present in warm rotting hay and similar substrates. Isolation is best made in plate culture using the glucose free medium to avoid too much overgrowth by other phycomycetes. The short brownish grey growth of *M. pusillus* is distinctive.

Candida albicans

C. albicans is a common pathogen of mammals, but less so in birds where it causes an oesophagitis. The soft lesions adhere weakly to the oesophageal ridges and cause a mild inflammation. In waterfowl the disease is usually found in birds debilitated by some other condition.

Caustic potash digests of scrapings from the linings of the oesophagus show a narrow pseudomycelium and yeast-like cells. Ink staining improves the visibility of the fungus but is slow and not intense.

Scrapings are inoculated into modified Czapek Dox agar stabs and incubated at 40° for 1–3 days. Any cream coloured yeast-like growth is subcultured on to Oxoid* Czapek Dox agar for purification and identification (Dawson, 1962). *C. albicans* cultures normally form characteristic thick-walled chlamydospores on this medium.

The fungus can be isolated from the living bird by using a rubber tube swab (Beer, 1963) which is pushed down into the oesophagus, removed and washed in 4 ml of the modified Czapek Dox broth in a bijou bottle. After incubation at 40° for 24 h. the broth is subcultured on to plates of Oxoid Czapek Dox agar.

* Medium CM 97 of Oxoid Ltd., Southwark Bridge Road, London, S.E.1.

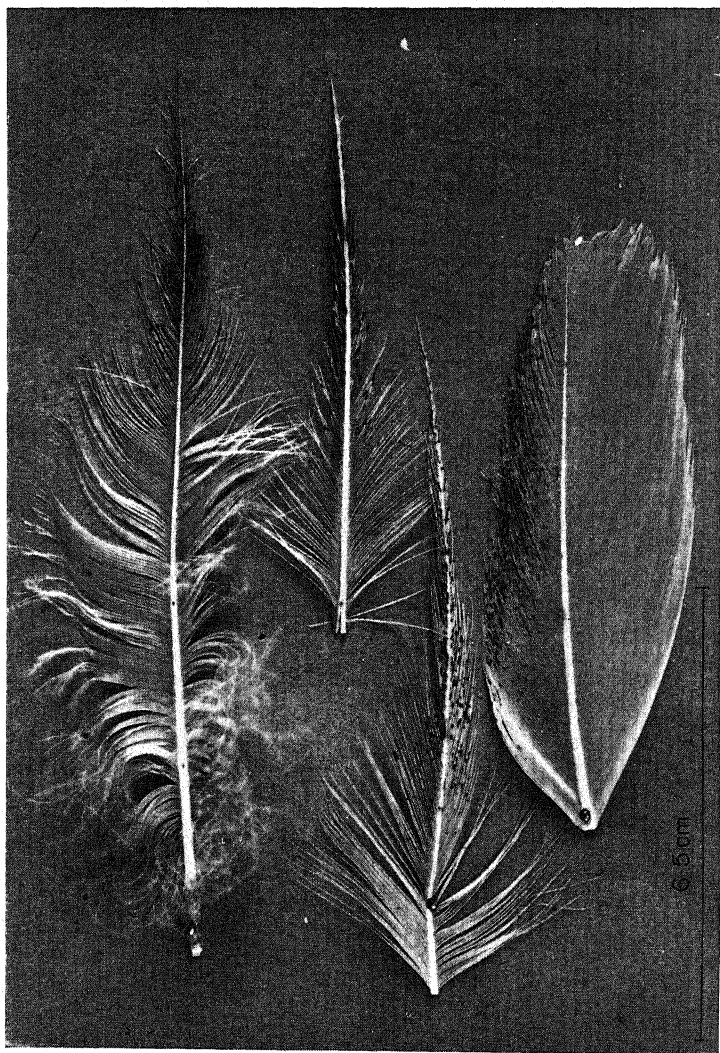


FIG. 4. Flamingo feathers with black sclerotia of *Cladosporium herbarum* causing early breakdown of the feather structure.



FIG. 5. Section of a feather barb with developing sclerotia composed of short-celled dark brown hyphae in the medulla.

The environment of birds may be contaminated by *C. albicans* (Keymer and Austwick, 1961) and the fungus can be isolated from washings by plating out on to the modified Czapek Dox agar and the Oxoid medium.

Cladosporium herbarum

Cladosporium spp. are commonly found on plants, on organic debris and in the air but very rarely cause disease in man or animals. *C. herbarum* is only known as an avian pathogen from the surface feathers of captive flamingos (Beer, unpublished data) although it is often found as a contaminant on birds' feathers (Pugh, 1965). It forms black sclerotia within the feather

rupturing the cortex and weakening the feather so much that the distal portions break off (Fig. 4).

The presence of hyphae can be seen in aqueous mounts but the nature of the growth can best be examined in 5% KOH digests, slightly warmed. Small, dark brown, interconnected sclerotia fill the medullary cell spaces (Fig. 5) and rupture the cortex forming short aerial hyphae. Hyphae in newly invaded cells are virtually colourless or very pale brown.

C. herbarum grows slowly even on a rich medium, and at its optimum growth temperature and pH. The Czapek Dox agar (modified) can be used but a richer medium is better for isolation from feathers.

Mycological peptone	10 g
Sucrose	10 g
Chloramphenicol	50 mg
Actidione	10 mg
Agar	15 g
Water, distilled	1 l
pH, adjust with HCl to 5	

Distribute into tubes or small bottles and sterilize at 115° for 10 min. Actidione is present to suppress the growth of yeasts which are present on many flamingo feathers.

Short lengths of feather barbs, containing the black sclerotia, are cut from the feather with sterile scissors, placed on a plate of medium and incubated at 22–24° for at least 5 days. Small, very dark smokey-green colonies, which are black in reverse, grow from the sclerotia. If contaminants are troublesome, temperatures below 10° can be used. However, growth is very slow and a culture will take a month or more to reach several millimeters in diameter. Subcultures are made on to malt agar and the fungus can be identified by its colonial form, dark brown, narrow, septate hyphae and branched chains of simple 1–4 celled spores (Gilman, 1957).

The isolation of this species from the environment presents some problems since a high incubation temperature cannot be used. In this instance it is better to use the modified Czapek Dox agar of higher selectivity at the optimum temperature or below 10° if the time factor is not important. If overgrowth has occurred, colonies of probable *C. herbarum* are easy to pick out because of their black reverse.

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Techniques for the Isolation of Pathogenic Fungi

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Fungi are involved in three types of diseases in man and animals.

First, *Infectious Diseases*. These mycoses are the result of the direct invasion of the tissue by the fungi, *e.g.*, *Aspergillus fumigatus* causing aspergillosis in man, birds and mammals; *Trichophyton verrucosum* causing human and cattle ringworm.

Second, *Toxicosis*. The mycotoxicoses which results from the ingestion of toxic metabolites, *e.g.*, *Claviceps purpurea*, the fungus causing ergotism.

Third, *Allergy*. The fungal allergies are not yet well defined in animals but much work has been carried out in the human field. These states are due to contact with fungal cells or products, usually through inhalation, *e.g.*, farmer's lung syndrome in man and fog fever in animals, both associated with the inhalation of thermophilic actinomycete spores from mouldy hay, straw and grain. One of the most common causes of Type I allergy in Britain is *Aspergillus fumigatus*. *Candida albicans* is also a known inducer of allergic states (Pepys *et al.*, 1967).

The methods used for the isolation of the causal agent from infected material varies with the specimen involved and the organism suspected. For this reason there are four sections to the discussion of techniques:

- (1) Isolation of fungi from deep mycoses;
- (2) Isolation of pathogenic yeasts;
- (3) Isolation of ringworm fungi (dermatophytes);

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(4) Air sampling and dilution plating for assaying fungal contents of air, grain, hay, straw and tissues.

Isolation of Fungi from Deep Mycoses

Deep mycoses: Many of these diseases evolve slowly and develop as chronic infections which persist for weeks, months and even years. These diseases are able to produce such a wide variety of clinical manifestations and should be considered when a differential diagnosis with questionable etiology is encountered. Cytotoxic drugs and steroids tend to weaken the natural competence of the body against proliferation by many of these organisms which are not generally primary invaders.

TABLE 1. *Diseases and causal organisms involved in deep mycoses*

Disease	Causal organisms
Actinomycosis	<i>Actinomyces bovis</i> , <i>A. israeli</i>
Nocardiosis	<i>Nocardia asteroides</i> , <i>N. brasiliensis</i> , <i>N. caviae</i>
Blastomycosis	<i>Blastomyces dermatidis</i>
Histoplasmosis	<i>Histoplasma capsulatum</i> , <i>H. farciminosum</i> , <i>H. duboisii</i>
Aspergilliosis	<i>Aspergillus fumigatus</i> , <i>A. terreus</i> , <i>A. flavus</i> , <i>A. niger</i> , <i>A. nidulans</i>
Mycetoma (1)	
Eumycetoma	<i>Allescheria boydii</i> , <i>Cephalosporum acremonium</i> , <i>Fusarium</i> sp., <i>Leptosphaeria senegalensis</i> , <i>Madurella mycetomi</i> , <i>M. grisea</i> , <i>Phialophora jeanselmei</i> , <i>Pyrenochaeta romeroi</i>
(2) Actinomycetoma	<i>Actinomyces bovis</i> , <i>A. israeli</i> , <i>Nocardia asteroides</i> , <i>N. madurae</i> , <i>N. pelletieri</i> , <i>Streptomyces somaliensis</i>
Chromoblastomycosis	<i>Phialophora verrucosa</i> , <i>P. pedrosoi</i> , <i>P. compacta</i> , <i>P. jeanselmei</i> , <i>Cladosporum carrionii</i>
Adiaspiromycosis	<i>Emmonsia crescens</i> , <i>E. parva</i>
Phycomycosis	<i>Absidia ramosa</i> , <i>A. corymbifera</i> , <i>Mucor pusillus</i> , <i>Rhizopus microsporus</i> , <i>R. oryzae</i> , <i>R. arrhizus</i>
Mycotic Abortion	<i>Aspergillus fumigatus</i> , <i>A. terreus</i> , <i>A. flavus</i> , <i>A. nidulans</i> , <i>Allescheria boydii</i> , <i>Absidia ramosa</i> , <i>A. corymbifera</i> , <i>Mucor pusillus</i> , <i>Mortierella</i> spp., <i>Rhizopus</i> spp., <i>Candida</i> spp.
Dermatomycosis (Ringworm)	<i>Arthroderma benhamiae</i> (<i>Trichophyton mentagrophytes</i>); <i>Epidermophyton floccosum</i> , <i>Microsporum audouinii</i> , <i>M. canis</i> , <i>M. distortum</i> , <i>M. equinum</i> , <i>Nannizzia fulva</i> (<i>Microsporum fulvum</i>), <i>Nannizzia gypsea</i> , <i>N. incurvata</i> (<i>Microsporum gypseum</i>), <i>Nannizzia obtusa</i> (<i>Microsporum nanum</i>), <i>Nannizzia persicolor</i> (<i>Trichophyton persicolor</i>), <i>Trichophyton concentricum</i> , <i>T. equinum</i> , <i>T. flavum</i> , <i>T. gallinae</i> , <i>T. megninii</i> , <i>T. quinckeanum</i> , <i>T. rubrum</i> , <i>T. sabouraudii</i> , <i>T. schoenleinii</i> , <i>T. soudanense</i> , <i>T. tonsurans</i> , <i>T. verrucosum</i> , <i>T. violaceum</i> .

Table 1 lists the organisms to be considered under the heading deep mycoses. It is important to realize that some members of the genus *Candida* and *Torulopsis*, discussed under the pathogenic yeasts, are also capable of causing disseminated infections. Many of the infections included in this list are generally considered to be endemic in origin. Today, travelling has increased to such an extent that many of the diseases, never considered in differential diagnoses outside the country of endemic origin, are being seen

TABLE 2. *Diseases and the media and incubation temperatures used to isolate causal organisms*

Disease	Media	Incubation temperature
Actinomycosis	Blood agar	37°
	Brain heart infusion glucose agar	37°*
	Brain heart infusion glucose broth	37°*
	Brewer's thioglycollate broth	37°
Nocardiosis & Actinomycetoma	Beef infusion blood agar	37°
	Beef infusion glucose agar	37°
	Glucose peptone agar	37°
Blastomycosis	Blood agar	37°
	Beef infusion glucose agar	37°
	Glucose peptone agar	25°
Histoplasmosis	Brain heart infusion glucose agar	37° & 25°
	Glucose peptone agar	25°
Aspergillosis	2% Malt agar	37° & 25°
	Glucose peptone agar	25°
Eumycetoma	2% Malt agar	37° & 25°
	Glucose peptone agar	25°
Chromoblastomycosis	2% Malt agar	25°
	Glucose peptone agar	25°
Adiaspiromycosis	2% Malt agar	37° & 25°
	Glucose peptone agar	37° & 25°
Phycomycosis	2% Malt agar	37° & 25°
	Glucose peptone agar	25°
Mycotic Abortion	2% Malt agar	37° & 25°
	Glucose peptone agar	37° & 25°
Dermatomycosis	Glucose peptone agar with cyclohexide & chloramphenicol	37° & 25°

* Incubated anaerobically with or without 5-10% CO₂.

throughout the world. Table 2 lists the diseases and the media used to isolate the causative agents.

Material to be cultured should reach the laboratory as soon as possible. Bacterial contamination often impedes the growth of these fungi. If contamination is present the specimens should be soaked in a penicillin-streptomycin solution (see appendix). Direct examination of exudates and sputum in 20% KOH often reveals the causative agent. Biopsy material is ground up and treated in the same manner. In suspected cases of histoplasmosis inoculation of mice intraperitoneally with 0.2 ml of the material, is often useful in isolation of the causative agent. The animals are sacrificed at one, two and three week intervals, and cultures are made from spleen and liver.

Biopsy material is placed into tubes of glucose peptone or malt agar with and without antibiotics. It is important to avoid using Petri dishes if *Histoplasma*, *Blastomyces* or *Coccidioides* is suspected since inhalation of spores from these organisms may be dangerous for the laboratory worker.

Material from pus is closely examined for the presence of grains or granules. These are picked out and washed several times in an antibiotic solution prior to plating, except in the case of *Nocardia* and *Streptomyces* mediated mycetomas. In such cases no antibiotic should be used when attempting to isolate these organisms, since they are sensitive to antibacterial agents.

Isolation of Pathogenic Yeasts

Yeasts are fungi in which the unicellular condition is predominant and reproduction is generally by budding. The most common yeasts associated with mycotic infections are members of the genera *Candida* and *Torulopsis* and *Cryptococcus neoformans*. The former are responsible for various clinical entities while the latter appears to have a predilection for the central nervous system; although pulmonary, dermal, osseous and visceral cryptococcosis do occur.

Infections caused by yeasts may occur in or on any part of the body. Yeasts are extremely easy to isolate. The difficulty arises when their significance must be assessed. Since many of these organisms may exist as saprophytes in or on many parts of the body it is imperative to confirm their presence by direct microscopy of the infected skin, nail, etc., or by histological sections of the biopsy material. The infections caused by yeasts may be:

- (1) superficial (localized or generalized)
- (2) chronic
- (3) disseminated.

*General methods for the isolation of yeasts**From skin*

This includes onychia, paronychia, vulvovaginal, mucocutaneous, infra-mammary, and other surfaces. The areas to be scraped or swabbed should be cleaned, being careful to remove any ointments or powders which may have been used by the patient. Scrapings and swabs are distributed into tubes of glucose-peptone agar with and without antibiotics. It is important to note that some yeasts capable of causing human infection are sensitive to actidione (cycloheximide). The tubes are incubated at 37°.

From sputum

Sputum specimens should be fresh. The diagnosis of bronchial or pulmonary candidiasis is most difficult to establish. Quantitative estimation of a large number of yeasts from repetitive fresh sputum specimens, without the presence of any other underlying disease might cause concern. However, the mere isolation of yeasts from sputum is hardly an indication of any pathological condition. The methods of obtaining the specimens should always include washing the mouth with some type of antiseptic prior to collection of the sputum. The sputum should be examined directly with 20% KOH for the presence of yeast cells and mycelium. Dilution plating should be done to give some indication of the number of organisms present.

If a primary pulmonary infection is suspected it is advisable to check the patient's serum for the presence of precipitating antibodies. Only the complete picture, clinical, cultural and serological can confirm a diagnosis of primary bronchial or pulmonary candidiasis, since these organisms are frequently present in sputum specimens, and almost always present in large numbers in cases of bronchogenic carcinoma, tuberculosis, bronchitis and patients undergoing antibiotic or steroid therapy.

From urine

Urine specimens should be catheterized to avoid contamination from the vaginal tract which normally harbours yeasts such as *Torulopsis glabrata* and *Candida albicans*.

Dilution plating is advisable, 10^5 yeasts/ml urine appears to be significant (Ahearn *et al.*, 1966).

From blood cultures

It is often difficult to isolate yeasts from blood in the case of vegetative lesions or deep seated infections. Eight to ten millilitres of blood are placed into flasks containing 100 ml of glucose-peptone broth and incubated at 37°. If systemic involvement is suspected it is advisable to have the patient's

serum tested for the presence of precipitating antibodies (Taschdjian *et al.*, 1967). Serological techniques are of value in the diagnosis of systemic candidiasis.

From cerebrospinal fluid (C.S.F.)

C.S.F. is centrifuged at 1500 g for 10 min and the sediment examined microscopically as well as inoculated into tubes of glucose-peptone broth. Direct examination of the C.S.F. with Nigrosin or India ink demonstrates the capsule of *Cryptococcus neoformans*. It is often difficult to isolate *C. neoformans* and other yeasts from the C.S.F.. Serological techniques have proved very reliable in the early diagnosis of cryptococcal meningitis. The test relies on the detection of cryptococcal antigen in the patient's serum or C.S.F.. This antigen titre of body fluids appears to reflect the hosts' yeast cell population, whether or not viable (Gordon and Vedder, 1966).

From biopsy specimens

Part of the specimen is ground up aseptically and plated, and part is fixed for histology. The organisms must be seen in the histological sections as active invaders to confirm the final diagnosis.

Isolation of Ringworm Fungi (Dermatophytes)

The ringworm fungi are a group of closely related species of the family Gymnoascaceae which have an affinity for the keratinized layers of the skin and its appendages (hair, feathers, nail and horn). The dermatophytes can sometimes be isolated from apparently uninfected animals when they are probably only present as part of the noninfective fungus population of the hair (For species list see Table 1).

A common habitat of keratinophilic fungi is the soil from whence, it is assumed, they originate living as saprophytes among other Gymnoascaceae.

These three sources of dermatophytes require different isolation techniques.

Isolation from lesions

Ringworm infected material often has characteristics which can be demonstrated using filtered ultra violet light (Wood's lamp) and a dissecting microscope. The greenish fluorescence seen under a Wood's lamp is produced by a complex organic substance formed by the action of certain dermatophytes (*Microsporum* species), on keratin. Most pathogenic dermatophytes parasitizing hair cause it to retain its root sheaths which can easily be seen under a dissecting microscope.

Method

1. Examine the sample for greenish fluorescence under a Wood's lamp, wavelength 3650 Angstrom units, and separate the fluorescent hairs, skin or scab material.

2. Using a dissecting microscope select any sheathed hairs.

3. At this stage a microscopical examination of the selected material is carried out. Mount the material in 20% KOH, on a slide under a cover slip, gently warm and examine under a compound microscope for arthrospores (a diagnostic characteristic) and hyphae.

4. Culture by planting several groups of the selected material in glucose-peptone agar containing antibiotics to suppress the growth of contaminating bacteria and fungi (see appendix).

5. Incubate for up to 4 weeks at 25° and 37° and any dermatophytes isolated are sub-cultured, identified, and kept if necessary, for reference purposes.

Isolation from apparently uninfected animals

The sampling of large areas of hair, fur and wool has been facilitated by the introduction of the hair brush technique (Mackenzie, 1963).

Method

1. Brush the hair with a sterile nylon brush.

2. The brush is stippled on the surface of glucose-peptone agar.

3. Incubate at 25° and subculture to enable pure isolations and identifications to be made.

Isolation from soil

The use of hair, wool, feathers and other keratinous material as "bait" to encourage the growth of dermatophytes in soil has led to the discovery of their presence in this environment.

Method

1. Sterilize hair, preferably human or horse, by autoclaving (121° for 15 min).

2. Mix with soil in a Petri dish.

3. Incubate at 25° for several weeks. An active colonization of the hair by keratinophilic fungi will result.

4. The hair is then removed and planted in glucose-peptone agar plus antibiotics and incubated as before.

Air Sampling and Dilution Plating for Assaying Fungal Contents of Air, Grain, Hay, Straw and Tissues

Air sampling

The sampling of the atmosphere to determine the number and types of fungi and actinomycetes present, is becoming more important in providing "background" information in the study of fungal diseases. There are several instruments available for this work, *e.g.*, the roto-rod sampler (Perkins, 1957), the Hirst spore trap (Hirst, 1952), the Cascade impactor (May, 1945) and the Andersen sampler (Andersen, 1958). The last of these examples is the main method used at Weybridge.

The Andersen sampler

The instrument is made from a light metal alloy and consists of a series of six stages through which the sample of air is drawn. The device is pressure sealed with gaskets in between each stage and three adjustable spring fasteners. Each stage contains a plate perforated with 400 holes of equal size and immediately below which is a Petri dish of agar medium. Air is drawn through the device at 20 litres/min and a jet of air from each of these holes plays onto the face of the medium. The holes in each stage are smaller than those in the preceding one, the largest at the top measuring 0.0465 in. diameter and the smallest at the bottom measuring 0.0100 in. diameter, the jet velocity therefore increases at each stage.

If one requires to know the number of viable spores, both fungal and actinomycete, four sets of six agar plates are used, two sets of one-half strength nutrient agar and two of malt agar. The sampler is "loaded" with six plates at a time; it is then attached to a vacuum pump and switched on after adjusting the airflow to 20 litres/min. The pump is run for 5 min, then the sampler is reloaded with fresh agar plates and the procedure is repeated for a total of four exposures. The one-half strength nutrient agar plates containing 0.5 mg/ml of cycloheximide to inhibit the growth of fungi, are incubated at 55° and 37°. The malt agar plates containing 20 units/ml of penicillin and 40 units/ml of streptomycin to inhibit bacteria and actinomycetes are incubated at 37° and 25°.

The results are recorded as the total number of colonies per set of plates or can be estimated by the positive hole method when the count is adjusted according to the table of Andersen (1958). For fungi the former method is preferred and for bacteria and actinomycetes the latter. These counts give the number of spores isolated from 100 litres of air.

Dilution plating: logarithmic dilutions

This method is used for hay, straw and grain samples.

Approximately 50 g of the material are weighed in a sterile closed container. This is to ensure that few spores are lost and also cuts down the risk of any infection or allergy in the operator. Dilute 1:10 w/v with sterile water and shake well. Make ten-fold dilutions, the limit set according to a microscopical count of the spores present, usually a limit of 10^{-6} is sufficient for normal material but dilutions up to 10^{-12} may be necessary for excessively mouldy samples. Make four series of dilutions by pipetting 1 ml of each into four empty Petri dishes beginning at the highest dilution. Pour into two sets of these approximately 15 ml cooled, molten agar containing 20 units of penicillin and 40 units of streptomycin per millilitre of medium (M+) and in the other two sets approximately 20 ml of cooled molten one-half strength nutrient agar containing 0.5 mg of cycloheximide per millilitre of medium (1/2 NA). Mix well before the medium sets, and then incubate according to Table 3 below.

TABLE 3

<i>Four sets of plates</i>				
Medium	1/2 NA	1/2 NA	M +	M +
Incubation temperature °	55	37	37	25
Period of incubation in days	3	5	5	7

The plates are examined after the appropriate incubation time recording the number and types of fungi or actinomycetes present. The result is given as the number of colonies per gram of material, specifying the number and types of fungi or actinomycetes present.

The evaluation of these results leaves much to be desired. A 10^5 or above count of actinomycete or fungal colonies per gram of material *could* cause infection or an allergic response in an animal or patient but the actual intake levels associated with any respiratory disease of fungal or actinomycete origin are unknown.

This logarithmic dilution method when used for grain samples only gives the number of viable spores on the surface of the grain—a more selective result may be obtained by separating the mouldy grains and carrying out direct cultures from cotyledonary material after sterilizing the surface with sodium hypochlorite solution (0.15% available chlorine). The result could be shown as the weight/percentage of grains affected by a specific fungus.

Dilution plating: direct dilutions

This method is used, mainly, to determine the viable spore content of lung material when an allergic condition viz. "Fog fever" or "Farmer's Lung Syndrome" is suspected.

Aseptically remove and weigh into a sterile bottle approximately 1.5 g of lung. Add the equivalent amount of sterile normal saline to give a 1/10 dilution. Homogenize the tissue in a Waring blender for 5 min or until the tissue has been completely broken down without any overheating taking place.

Dispense the appropriate amount into 16 empty Petri dishes, add the cooled, molten medium, mix, allow to set and incubate as follows:

Medium	volume ml				Temperature °	Period days
M+	1	0.5	0.25	0.1	25	7
M+	1	0.5	0.25	0.1	37	5
1/2NA	1	0.5	0.25	0.1	37	5
1/2NA	1	0.5	0.25	0.1	55	3
Dilution	1/10	1/20	1/40	1/100		

The plates are read after the appropriate incubation period and the result recorded as the number of colonies per gram of lung, specifying the types of fungi and actinomycetes present. This is determined by adjusting the count on each plate to colonies per gram and taking an average of the counts on four plates at each temperature.

The evaluation of these results is made more difficult by the lack of experimental data showing the concentrations at which the various types of spores can cause disease.

Appendix*Media**Brewers Thioglycollate Broth*

Lab Lemco	10 g
Peptone	10 g
Sodium chloride	5 g
Sodium thioglycollate	1 g
Agar	0.5 g
Glucose	to 10 g
Methylene blue	0.002 g (1 in 500,000)

Distilled water 1000 ml

This is usually used in the dehydrated form as supplied by Oxoid.

Brain Heart Infusion Glucose Agar

Calf Brain	200 g
Beef Heart	250 g
Peptone	10 g
Glucose	2 g
Sodium chloride	5 g
Disodium phosphate	2.5 g
Distilled water	1000 ml

pH 7.4

Add 1.5% agar, if the solid medium is required.

This is usually used in the dehydrated form as supplied by Difco.

Beef Infusion Broth

Five hundred grammes of fat free beef. Mix with 1 litre of distilled water and leave overnight at 4°. Strain through muslin, press, steam for 1 h to coagulate the protein and filter to clear. Sterilize 121° for 15 min. Add peptone 1%, sodium chloride 0.5%. Raise to pH 7.8 to 8.0 with Sodium hydroxide. Steam for 15 min to dissolve components, filter and adjust to pH 7.5.

Beef Infusion Blood Agar

Beef infusion broth plus 1.5% agar and 5% sheep blood (added aseptically).

Beef Infusion Glucose Agar

Beef infusion broth plus 1.5% agar and 1% glucose (added aseptically).

Blood Agar

Peptone (Evans)	10.0 g
Lab. Lemco (Oxoid)	5.0 g
Sodium chloride	5.0 g
Agar (Davis)	15.0 g
Distilled water	1000 ml

The Lemco is boiled separately in a bowl to precipitate phosphates by addition of caustic soda (N NaOH to pH 8.0). It is then filtered through folded filter paper (Whatman No. 52), and added to the other ingredients. Distilled water is added and the pH is adjusted to 7.8–8.0. Autoclave at 128° for 5 min. Filter through paper pulp, adjust pH to 7.5 and distribute. Sterilize at 121° for 20 min; add 5% sheep or horse blood immediately before pouring plates at 57°.

Nutrient Broth

Bacto peptone (Difco)	10.0 g
Lab. Lemco (Oxoid)	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml

Precipitate phosphates in usual manner and filter. Adjust pH to 7.5. Sterilize at 121° for 15 min.

Nutrient Agar

Add 1.5% agar (Davis) to the broth.

Half Strength Nutrient Broth

Bacto peptone (Difco)	5.0 g
Lab. Lemco (Oxoid)	5.0 g
Sodium chloride	2.5 g
Distilled water	1000 ml

Precipitate phosphates in the usual manner and filter. Adjust pH to 7.0. Sterilize at 121° for 15 min.

Half Strength Nutrient Agar

Add 1.5% agar (Davis) to the broth.

Glucose Peptone Agar

Glucose	40.0 g
Peptone (Difco)	10.0 g
Agar (Davis)	15.0 g
Distilled water	1000 ml

Dissolve the peptone and agar by heating, then add the glucose. Sterilize at 108° for 15 min. Add aseptically 10 ml of Seitz-filtered 30% yeast extract (Difco) solution on pouring.

Use—this is the standard medium for the isolation and identification of dermatophytes and is sometimes useful for other pathogens. Antibiotic supplementation is necessary to suppress the growth of bacteria and of saprophytic moulds on primary isolation plates. Penicillin and streptomycin or chloramphenicol may be added as under malt agar. Cycloheximide should be added to media for dermatophyte isolation.

Malt Agar

Malt extract	10 g
Agar	20 g
Distilled water	1000 ml

Sterilize at 115° for 15 min.

*Antibiotic supplements**Penicillin and Streptomycin*

Twenty units of penicillin and 40 units of streptomycin are generally sufficient to suppress bacterial growth. The stock solutions are prepared as follows.

Penicillin

Add 20 ml sterile physiological saline to a phial containing 200,000 units crystalline potassium penicillin, giving a concentration of 10,000 units per ml. Take 10 ml of this and add to 40 ml of saline to give a stock solution containing 2000 units per ml. Add 1 ml of this to 100 ml cooled medium to obtain 20 units per ml final concentration.

Streptomycin

Dissolve 1 g (1 million units) in 20 ml sterile saline to give a solution of 50,000 units per ml. Take 4 ml of this and add to 46 ml sterile saline. This forms the stock solution containing 4000 units per ml. Add 1 ml stock solution to 100 ml cooled medium to obtain final concentration of 40 units per ml.

Chloramphenicol (Chloromycetin—Parke-Davis)

0.05 mg per ml of medium suppresses bacterial growth in agar and the antibiotic may be added before autoclaving.

Cycloheximide (Actidione—Upjohn)

Place 250 mg crystalline cycloheximide in a 50 ml volumetric flask. Add 5 ml to 10 ml acetone to dissolve, then bring to volume with distilled water (pH adjusted to 6.5–6.8). Sterilize by autoclaving at 120° for 15 min and store at 5°. This solution will remain stable for 5–6 weeks. Add 10 ml stock solution to 100 ml cooled medium to obtain 0.5 mg per ml final concentration.

Note—cycloheximide is extremely toxic and causes severe irritation of the skin and mucous membranes (see instructions around bottle). It should not be used in media for pathogenic mould fungi or yeasts, but can be included in media for the isolation of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Sporothrix schenckii* and *Coccidioides immitis*. No attempt however, should be made to isolate *Histoplasma* or *Blastomyces* at 37° with cycloheximide since the yeast phase of these organisms is sensitive to this antibiotic.

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Membrane Filtration Techniques for the Isolation from Water, of Coli-aerogenes, *Escherichia coli*, Faecal Streptococci, *Clostridium perfringens*, Actinomycetes and Microfungi

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The membrane filtration techniques described are mostly adaptations of other isolation procedures. The use of membranes facilitates the concentration of organisms from relatively large volumes of water in which particular species may be sparsely distributed and it often enables a greater variety of species or types to be isolated, especially when compared with tube or enrichment methods in which usually only the predominant types are isolated. Most methods require considerable modification of media when adapted for membranes. Some of the methods have been described elsewhere (Burman, 1967*a*).

Materials and Equipment

All the work described has been carried out using the 50 mm Gallenkamp membrane filter apparatus with 47 mm Oxoid membrane filters. The apparatus has a cylindrical aluminium funnel graduated at 50 and 100 ml or alternatively a larger conical funnel, clamped to the filter base via silicone rubber gaskets surrounding a porous carbon or sintered glass disc. The use of sintered bronze discs is not recommended. The apparatus was designed to take 50 mm membranes but it is easier to manipulate 47 mm membranes.

New membranes are sterilized by interleaving with Whatman no. 1 filter paper, packing tightly in a Petri dish and boiling, unless they are being used with a non-selective medium in which sporing organisms might interfere, in which case they should be autoclaved according to the maker's instructions. Overheating, however, often causes changes in filtration characteristics.

For some purposes repeated re-use is permissible. These and other general membrane procedures have been described by Burman (1967*b*) and Windle Taylor and Burman (1964).

Where liquid media are described, these are used to saturate a 47 mm Whatman no. 17 filter paper in a Petri dish. A slight excess of medium is added and the surplus poured off immediately before placing the membrane.

Owing to the relative adsorption of the various compounds which make up a complex culture medium, between different types of materials such as the membrane and the absorbent pad or the agar, no guarantee can be given that similar results will be obtained if different makes of membranes or pads are used or if an agar medium is substituted for an absorbent pad or *vice versa*.

For accurate temperature control in a water bath, disposable polystyrene dishes are placed in a heavy cylindrical brass container with screw-on lid and breathing tube (Astell Laboratory Service Co. Ltd) which is completely immersed in the bath. To prevent drying out during incubation in an ordinary air incubator, the dishes are placed in polythene boxes with tightly fitting lids.

Isolation of Coli-aerogenes

The coli-aerogenes group is a heterogeneous group with a wide range of optimum temperature and growth requirements. Membrane filtration procedures can be modified to isolate any section of them. The water supply bacteriologist is concerned mainly with those capable of producing acid and gas from lactose in 48 h at 37°. The media recommended for this purpose are as follows (Burman, 1967b).

3% Teepol broth (3T): peptone (Oxoid), 10 g; lactose, 30 g; NaCl, 5 g; Teepol 610 (BDH), 30 ml; 0.4% aqueous phenol red, 50 ml; water to 1000 ml; pH 7.4.

3% Enriched adsorbed Teepol broth (3 EAT): peptone (Oxoid), 40 g; yeast extract (Oxoid), 6 g; lactose, 30 g; Teepol 610 (BDH), 30 ml; 0.4% aqueous phenol red, 50 ml; water to 1000 ml; pH 7.4. The peptone yeast extract mixture is adsorbed with 10 g granular cellulose triacetate (Oxoid) overnight and then filtered.

The 3 T and 3 EAT should be sterilized by steaming for 30 min on each of three successive days. The pH will fall and should be 7.4 after sterilization so that allowance for this must be made by making the initial pH 7.8.

Dilute resuscitation broth (DRB): peptone (Oxoid), 0.1 g; yeast extract (Oxoid), 0.015 g; lactose, 0.075 g; $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, 0.71 g; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.16 g; water to 1000 ml, sterilized by steaming for 30 min on each of three successive days.

To obtain a count approximately equivalent to that obtained by the standard MacConkey broth multiple tube count in 48 h at 37° on unchlorinated water, the 3 T medium is recommended. A higher count can be obtained and a greater variety of types isolated using the 3 EAT medium.

Incubation on either of these media should be at 30° for 4 h followed by 35° for another 14 h. For samples likely to contain attenuated or damaged organisms incubation at 25° for 6 h followed by 18 h at 35° is recommended. The temperature changes can be achieved with automatic timed equipment.

To obtain the most organisms from samples which have been treated, especially by chlorination, a resuscitation technique is recommended using dilute resuscitation broth (DRB). Incubation should be at 25° for 18–24 h on DRB followed by transfer to 3 EAT at 35° for 6–24 h. The optimum times of incubation on each medium are still under investigation. Highest results are obtained with the longest incubation times for chlorinated water, but with unchlorinated water overgrowth with non-coliform organisms is liable to occur.

Isolation of *Escherichia coli*

The media used for isolation of *E. coli* are similar to those used for coli-aerogenes except that a lower concentration of Teepol is used in conjunction with a temperature of 44°.

0.4% Enriched adsorbed Teepol broth (0.4 EAT); same as 3 EAT but with 0.4% Teepol 610 instead of 3%. Incubation is at 30° for 4 h followed by 44° for 14 h; or 25° for 6 h followed by 44° for 18 h for chlorinated samples. A positive result can usually be obtained in 8–12 h total incubation time. As with the coli-aerogenes, higher results can usually be obtained on marginally chlorinated samples by incubating for the first 18–24 h on DRB at 25° followed by transfer to 0.4 EAT at 44° for 6–24 h. Optimum times are still under investigation.

Isolation of Faecal Streptococci

The media recommended are as follows.

Slanetz and Bartley enterococcus agar (SBA): tryptose (Difco or Oxoid), 20 g; yeast extract (Difco or Oxoid), 5 g; dextrose, 2 g; K_2HPO_4 , 4 g; sodium azide, 0.4 g; triphenyl-tetrazolium chloride (TTC), 0.1 g; agar (Oxoid), 10 g; water to 1000 ml; pH 7.2 (Slanetz and Bartley, 1957). All the ingredients except the TTC are steamed to dissolve. The TTC is then added as a sterile 1% solution and the medium is poured directly into Petri dishes without further heating.

Mead's tyrosine sorbitol thallos acetate agar (TSTA): peptone (Oxoid), 10 g; yeast extract (Oxoid), 1 g; sorbitol, 2 g; tyrosine, 5 g; agar (Oxoid), 12 g; water to 1000 ml. The medium is autoclaved at 115° for 10 min, adjusted to pH 6.2 followed by addition of TTC, 0.1 g; thallos acetate, 1 g; tyrosine, 4 g; and plates immediately poured. The basal medium after autoclaving may be stored in 100 ml volumes and appropriate quantities of

the other ingredients added to whatever quantity is required. Most of the initial quantity of tyrosine dissolves on autoclaving, the extra tyrosine added later remains in suspension. Double layer plates are more satisfactory, the lower layer basal medium only and the upper layer complete medium (Mead, 1963).

Slanetz and Bartley (1957) recommended incubation on SBA at 36° for 48 h and claimed that all the streptococci isolated could be classified as enterococci. This has not, however, been found to be the case with water samples in Great Britain, particularly with river waters (Burman, 1961). Many unclassifiable strains can be isolated by this method. With incubation at 36–37° for the first 4 h followed by 44 h at 44–45°, however, counts have been obtained equivalent to those obtainable with a multiple tube test, and the majority of the strains isolated belonged to Lancefield Group D (Mead, 1966).

A further differentiation of *Strep. faecalis*, *sensu strictu* can be made by subculture on Mead's (1963) TSTA medium. Mead found that this organism was associated more frequently with faecal pollution from man than from animals, especially herbivores (Windle Taylor, 1963–1964). Best results can be obtained by subculturing individual colonies. *Strep. faecalis*, *sensu strictu* will produce dark maroon colonies surrounded by a clear area in three days at 44°. A much quicker assessment can be made by an imprinting technique. The membrane from the 48 h, 37° culture on Slanetz and Bartley agar is inverted on TSTA medium and immediately removed. After incubation for 4 h at 37° followed by three days at 44° clear areas will develop round colonies of *Strep. faecalis*, *sensu strictu*. Not much attention can be paid to the maroon colour of the colonies, because sufficient quantity of the original coloured colony is transferred to interfere with this. This can be overcome by omitting TTC from the SBA medium used for the initial culture.

To obtain the best results, the imprinting technique must be carried out with some care. The plates of TSTA medium should be well dried and the membrane with the primary culture should preferably be drained by placing it on the base of a membrane filter apparatus and applying a vacuum for a short time.

Isolation of *Clostridium perfringens*

A simple adaptation of technique using Wilson and Blair glucose sulphite agar (Report, 1956) gives results with membrane filters comparable to multiple tube litmus milk tests. The medium is made up as follows: nutrient agar with 2% agar (Oxoid), 100 ml; 20% solution Na₂SO₃, 10 ml; 20% solution glucose, 5 ml; 8% solution FeSO₄·7H₂O (freshly prepared), 1 ml. The nutrient agar is sterilized at 121° for 20 min. The other solutions are

separately sterilized and added to the melted agar immediately before pouring into Petri dishes. The water sample is heated for 10 min at 75–80° before filtration. The membrane is placed face down on the surface of well-dried poured plates and another layer of the medium is poured on top. Incubation is for one to five days at 37–45°. *Clostridium perfringens* will produce colonies with large black halos.

The method has the advantage that quantities of 100 ml or more can be concentrated on one membrane if only low numbers are being sought. If the extent of pollution is unknown a series of doubling dilutions rather than ten-fold dilutions should be set up, as the size of the halos will quickly produce a confluent blackened area with relatively small numbers of colonies.

Isolation of Actinomycetes

Most of the techniques and media for isolation of actinomycetes have been designed for their isolation from soil. Isolation from water presents quite different problems however, as the ratio of bacteria to actinomycetes is usually much higher in water than in soil, so that a much more selective technique is required. The most suitable medium to use in conjunction with a membrane filtration technique has been found to be the chitin agar of Lingappa and Lockwood (1962), containing chitin as the sole source of C and N. It is prepared as follows: K_2HPO_4 , 0.7 g; KH_2PO_4 , 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4$, 0.001 g; agar (Oxoid), 10 g; distilled water, 1000 ml; pH, 7.0; sterilized at 121° for 20 min. To each 100 ml of melted agar is added 20 ml of colloidal chitin suspension and sufficient actidione (Koch–Light Co. Ltd) to give a final concentration in the medium of 50 µg/ml. Other antifungal antibiotics such as fungizone or nystatin may be used if preferred with equal success. Double layer plates are used, the first layer consisting of the basal medium with actidione but without the chitin. When this is set the second layer is poured with actidione and chitin. The purification and preparation of the colloidal chitin suspension is time-consuming and requires some care. The following procedure will give satisfactory results. Crude colloidal chitin must be washed alternately for 24 h at a time with N NaOH and N HCl about five or six times each. The mixture should be allowed to settle each night and the supernatant poured off each morning. It is then washed three or four times with 95% ethanol. This removes about 40% of the crude material and leaves a white product. It is advisable to treat 10 to 20 g at one time. If a pure white chitin can be obtained, this preliminary washing treatment is unnecessary. The clean white chitin is then treated with sufficient concentrated HCl to form a stirrable mixture, (about 100 ml to 15 g) and this is stirred for 20 min in an ice bath. The resultant mixture is centrifuged. The supernatant is

poured into cold distilled water when the chitin will be precipitated. The deposit is treated with further concentrated HCl and the process repeated until no further precipitate is obtained on adding to cold water. The concentrated acid chitin mixture must not be left standing. The whole acid treatment process must be completed in one working day otherwise the chitin will be degraded.

The chitin precipitate can be allowed to settle overnight and the supernatant decanted. The remaining suspension should then be centrifuged and washed and recentrifuged two or three times. The final deposit should then be mixed with sufficient distilled water in a blending machine to give a suspension with a creamy consistency which just pours easily. This suspension is then neutralized with NaOH to pH 7.0, distributed in approximately 20 ml amounts in wide mouth 1 oz universal screw-capped containers and sterilized at 121° for 20 min.

The above process requires a fairly large centrifuge preferably capable of taking 4×250 ml buckets. If such a machine is not available, filtration of the suspension through glass fibre filter paper on a buchner funnel is possible.

As chitin is insoluble in water it cannot diffuse through a membrane, so it would not be available to organisms on the membrane surface. The membrane must, therefore, be placed face downwards on the surface of the well-dried medium. This should be incubated at 22–30° for 4 h and then carefully removed and discarded and the culture returned to the incubator for 1–8 weeks. The organisms imprinted on the surface will then grow as normal surface colonies. Antibacterial antibiotics, namely penicillin and polymyxin B, sometimes recommended for soil, have been found to be ineffective with water samples and even inhibitory to some strains of actinomycetes.

The method can be made much more selective by treating the sample first with chloramine. Excess ammonia, as 0.2 m. of a sterile 0.38% solution of ammonium chloride, is added to 100 ml of water sample at room temperature to which is then added 1 ml of a hypochlorite solution containing 200 mg/l of available chlorine, to give a concentration of 2 mg/l in the sample. This is allowed to stand for 10 min after which the chlorine is neutralized with 0.05 ml of a sterile 3% solution of sodium thiosulphate. The sample is then filtered in the normal manner. The success of this method varies with the sample, but it is capable of suppressing many of the interfering bacteria, resulting often in much higher counts of actinomycetes, greater variety of species and much easier isolation of pure cultures uncontaminated with other bacteria.

Preliminary trials still under investigation have also shown that in many instances even more selective results can be obtained by heating the water

sample before filtration. Heating for 1 h at 44° has proved very effective but 1 h at 50° appears to be too inhibitory. Lower temperatures and longer and shorter times are still under investigation. A combination of chlorination and heating is also possible.

Some common *Streptomyces* and *Micromonospora* grow readily in 7–14 days at 30°. A much greater variety of species is obtained by prolonged incubation at lower temperatures particularly it seems with chlorinated or heated samples. Interference from other bacteria is greater at 22° than at 30°. Optimum times and temperatures are still under investigation but these will undoubtedly vary for different species and different samples due to variation in the associated microflora.

Colony appearance is not very typical on chitin agar and final isolation and purification is made by subculture to a more nutrient medium such as starch casein agar (Kuster and Williams 1964; Williams and Davies, 1965). Species of *Streptomyces*, *Streptosporangium*, *Micromonospora* and *Nocardia* have frequently been isolated from water by these methods.

Isolation of Fungi and Yeasts

The methods described were designed to isolate fungi responsible for the production of earthy, mouldy or musty tastes in water, due to growth in long lengths of piping under warm conditions (Burman, 1965). There are other fungi present in natural waters which require other specialized methods for their isolation (Willoughby, 1962).

The medium recommended is acid rose bengal kanamycin agar which is a modification of a medium introduced by Martin (1950). It is prepared as follows: mycological peptone (Oxoid), 5 g; dextrose, 10 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; rose bengal, 0.7 g; agar (Oxoid), 10 g; water to 1000 ml; pH 5.4, sterilized at 115° for 10 min. Kanamycin to give a final concentration of 100 µg/ml is added before pouring plates.

After filtration, membranes are placed face upwards in the normal manner and incubated at 22° for seven days. The medium is highly selective for fungi and yeasts. Aerobic sporing bacilli occasionally produce a few small colonies which are easily recognizable. The rose bengal suppresses common rapidly growing spreading fungi so that slow growing species can be readily isolated.

Species which have been readily isolated in association with mouldy tasting water include *Cephalosporium* sp., *Verticillium* sp., *Trichoderma sporulosum*, *Nectria viridescens*, *Phoma* sp. similar to *P. eupyrena*, and *Phialophora* sp. similar to *P. fastigiata*. We are indebted to the Commonwealth Mycological Institute, Kew, for the identification of these cultures.

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Isolation of Surface Micro-organisms with the Agar Slice Technique (Agar Sausage)

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The technique of using slices of agar medium cut from an agar sausage, to isolate surface organisms, was first described by ten Cate (1963, 1965).

The agar sausage technique soon acquired acceptance for the control of plant hygiene (Kraus and Landgraf 1964, Rozier 1964). Zipplies (1965) described the use of the sausage in the hygienic control of carcasses and other meat products, during the handling in meat industry, pointing out the advantages over swabbing and scraping techniques.

The efficiency of this technique in the hygienic control of food premises was described by Grieg (1966) and its value in the comparison of preparation surfaces and cleaning methods in the meat industry described by Mossel *et al.* (1966) and Cooper and Dyett (1967).

The method of preparation of the agar sausage, described by ten Cate (1963) was not without its difficulties and held back full development of the technique. An improved method of manufacture has now made the agar sausage commercially available.

Description of Sausage

The agar sausage ("Agaroid" Oxoid) is a sterile agar medium, enclosed in plastic tubing measuring 19 cm long by 3.3 cm diameter (8.5 sq. cm area).

The following media are available at the moment from Oxoid Ltd, Southwark Bridge Road, London, S.E.1:

Plate Count Agar
MacConkey Agar
Mannitol-Salt Agar
Sabouraud Maltose Agar
Malt Extract Agar

Sampling Technique

(1) Swab the outside of the casing with alcohol in the area to be cut and sterilize a very sharp, broad-bladed knife by swabbing with alcohol and flaming.

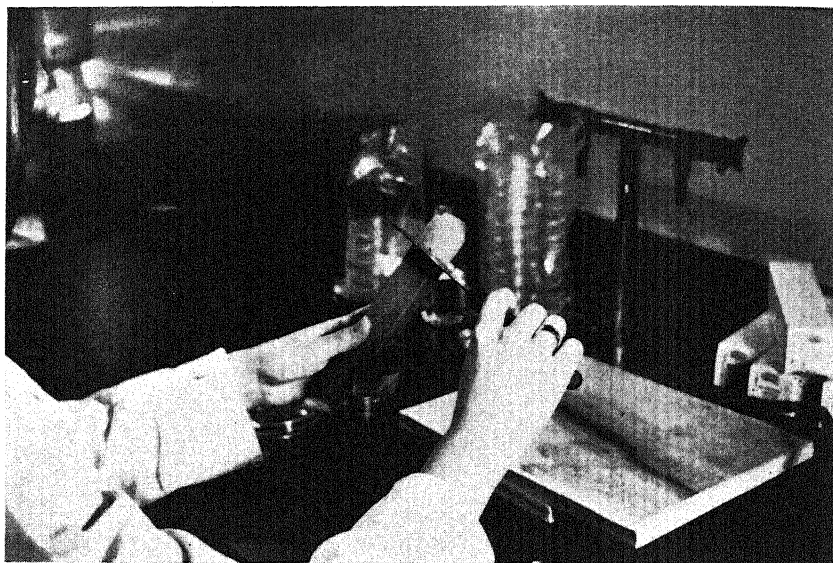


FIG. 1. The end of the agar sausage being removed.

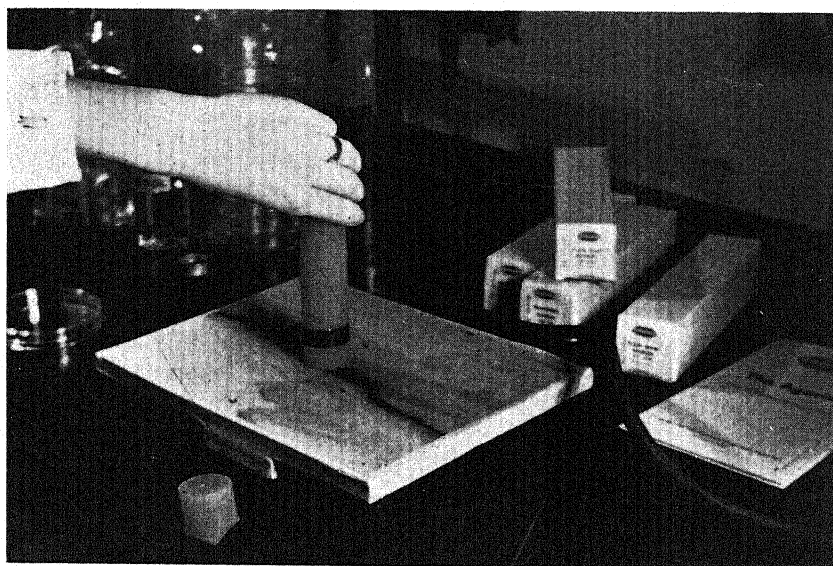


FIG. 2. The extruded cut end pressed firmly against the test surface.

- (2) Cut off the end of the agar sausage approximately half an inch (1 cm) distal to the coloured band. (Fig. 1). Do not remove the coloured band: it is provided to prevent the agar slipping out of its casing during use.
- (3) Applying light pressure at the base, push out about one centimeter of the agar column.
- (4) Sample by pressing the cut end of the agar firmly onto the test surface. (Fig. 2).
- (5) Cut off a slice 4–6 mm thick and transfer, inoculated side uppermost, to a Petri dish by supporting the agar slice on the blade of the knife (Figs. 3 and 4). Do not put the exposed end of the Agaroid down on a bench or other contaminated surface. Once the plastic casing has been cut do not re-seal. It is seldom necessary to re-sterilize the knife between successive slices taken from the same area or product.
- (6) Incubate the agar slices in closed Petri dishes at 37° for 18–24 h. Four slices may be placed, exposed side uppermost, in a 9 cm dish. The bacterial flora of the test surface can be determined by examination of the colonies on the agar. (Fig. 5)
- (7) Sterility controls may be made randomly, during the sampling, by merely cutting unexposed slices of medium and incubating them.

Discussion

If the sampling area does not require the whole agar sausage, then the medium can be slipped back into the casing, the top closed and the operator can move on to the next area. It is unlikely, however, that satisfactory results will be obtained from opened agar sausages that have been held overnight.

It is important that the cut agar surface is pressed and not wiped against the sampled area. This will allow a duplicate image of the microbial flora to be developed on the incubated slice of medium. The replication is especially valuable when sampling cracked surfaces, thus showing evidence of organisms in the cracks.

A wide variety of surfaces may be tested, using this technique. For example, carcase meat and abattoir instruments, skin surfaces of the body, leaves of plants, shells of eggs and fabrics. It should, however, be noted that the agar sausage method is not successful on very wet surfaces.

The agar slices will not give exact counts of micro-organisms present on the surface because of factors such as bacterial clusters (micro-colonies) and non-adhering organisms. Nevertheless, if sufficient samples are taken comparative values can be obtained as the errors in the method are randomly distributed. (Zipplies, 1965; Charnley and Eftekhari, 1969.)

Experience quickly determines acceptable levels of growth for each

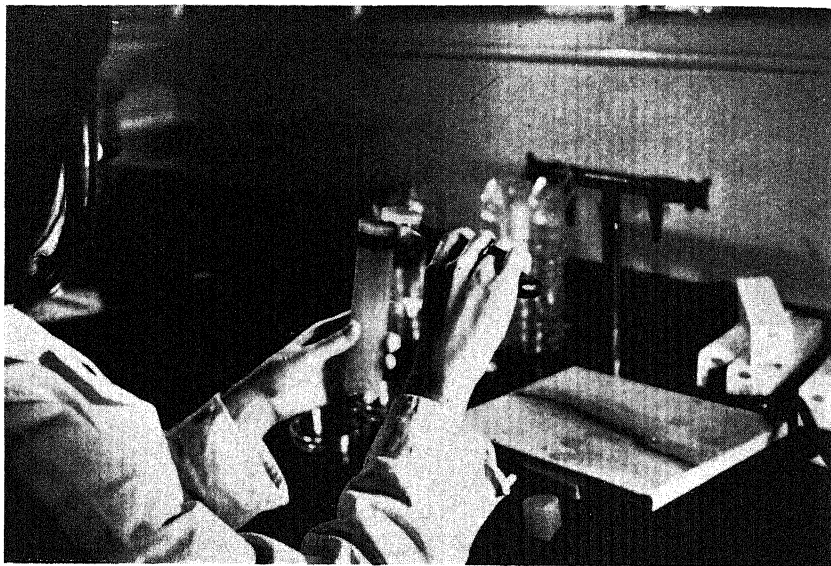


FIG. 3. The exposed end sliced from the sausage.

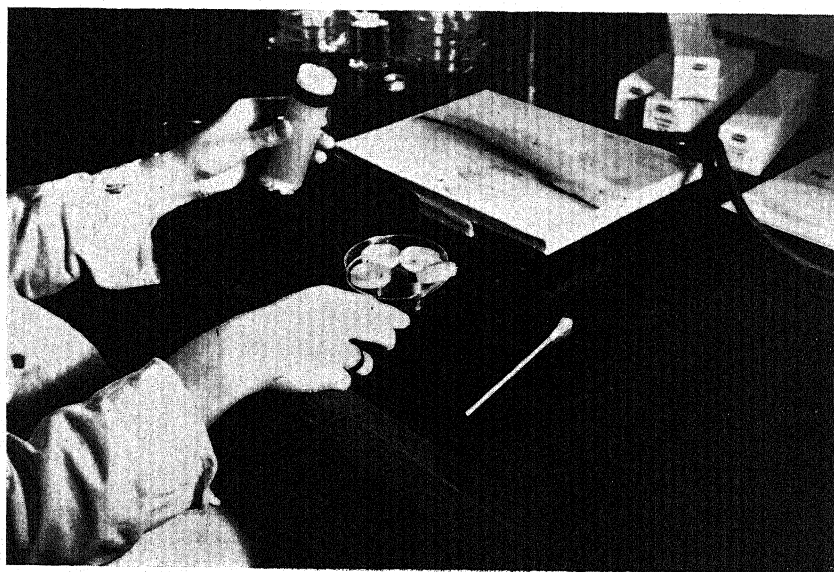


FIG. 4. Slipping the agar slice into the Petri dish.

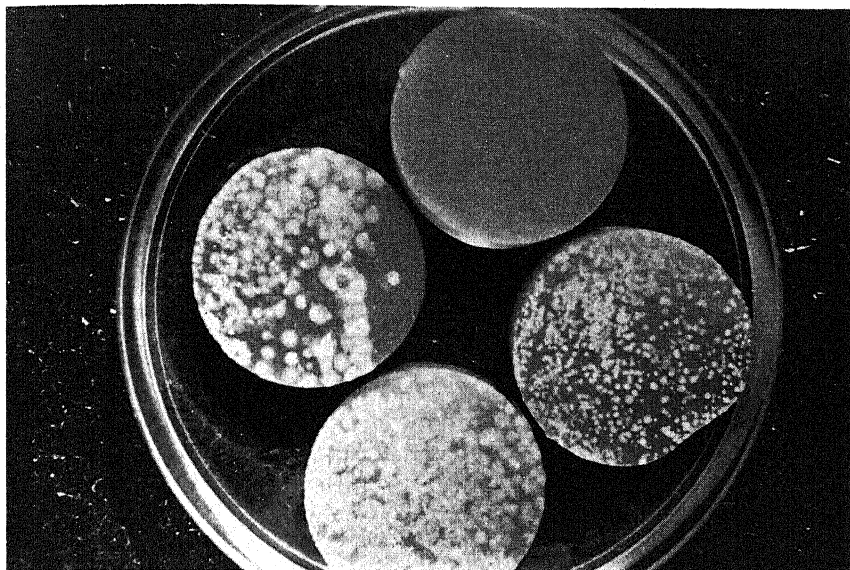


FIG. 5. Incubated agar slices showing growth of bacterial colonies and sterile control slice.

particular product; ten Cate (1965) used the following criteria to recognize the levels of contamination:

1. No growth
2. Very slight growth (10 colonies) \pm
3. Slight growth (10-30 colonies) +
4. Moderate growth (30-100 colonies) ++
5. Heavy growth (100 colonies) +++
6. Confluent growth ++++

Zipplies (1965) suggested the following enumeration:

1. 0-10 colonies
2. 10-100 colonies (estimated to the nearest 10)
3. 100-1000 colonies (estimated by counting colonies in a known area with a stereomicroscope)

Differential media can be used to estimate the microbial ecology of the area under examination, including yeasts and moulds.

If the agar sausage is to be used to detect the efficacy of disinfection, then specific neutralizers should be present in the agar. At present, development of media containing neutralizers such as Lubrol and Tween is being made with specialized applications of the agar sausage.

Finally, an important practical Quality Control use of this technique is that samples can be taken by Supervisory staff and incubated in their offices overnight. Demonstration of the resulting flora to process operatives brings home the importance of hygiene and cleanliness in their activities.

Acknowledgement

The author is indebted to Dr L. ten Cate for the following extensive list of references to uses of the agar sausage technique.

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Some Isolation Techniques Used in the Evaluation of Antibacterial Compounds

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Isolation techniques, including replica plate methods, are of considerable use in the evaluation of new compounds for antiseptic or chemotherapeutic use.

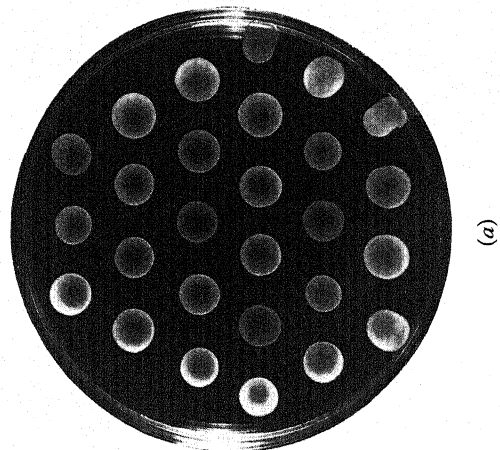
Replica Techniques

Replica plating methods can be employed *in vitro* to study the resistance of bacteria to new antibacterials and to measure the bactericidal activity of new compounds. Replica methods are also used to determine if organisms have been eradicated from skin or artificially infected wounds by treatment with formulated antibacterial compounds.

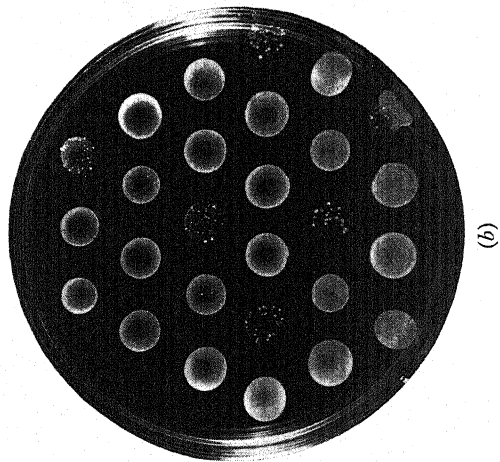
Drug resistance

Lederberg and Lederberg (1952) devised a simple but effective technique for copying a pattern of microbial growth from one initial agar plate to a series of others. This was accomplished by using velvet or velveteen to make transfers without disturbing the spatial relationships of the colonies. Using this technique to make replicas on agar containing streptomycin Lederberg and Lederberg showed that mutants of *E. coli* resistant to the antibiotic existed in clones on an initial plate of agar not containing streptomycin. The mutants were concentrated by taking inocula from sites demonstrated to contain them by replica plating and, after several stages of enrichment, pure cultures of the resistant mutants were obtained without exposing them at any time to streptomycin. This technique has found wide application to genetic studies and is also of use in the study of chemotherapeutic agents.

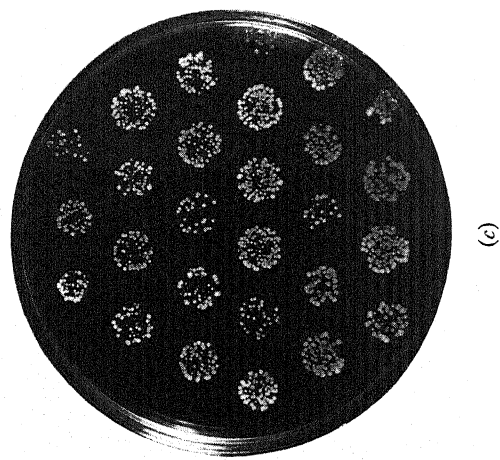
When a minority of cells in an otherwise sensitive population of bacteria are insensitive to an antibiotic sharp end-points are not seen in agar-dilution tests. The iron-containing antibiotics known as ferrimycins (Bickel *et al.*, 1960) are notorious for exhibiting this phenomenon (Fig. 1) which has hindered the practical use of these highly active agents. We have employed



(a)



(b)



(c)

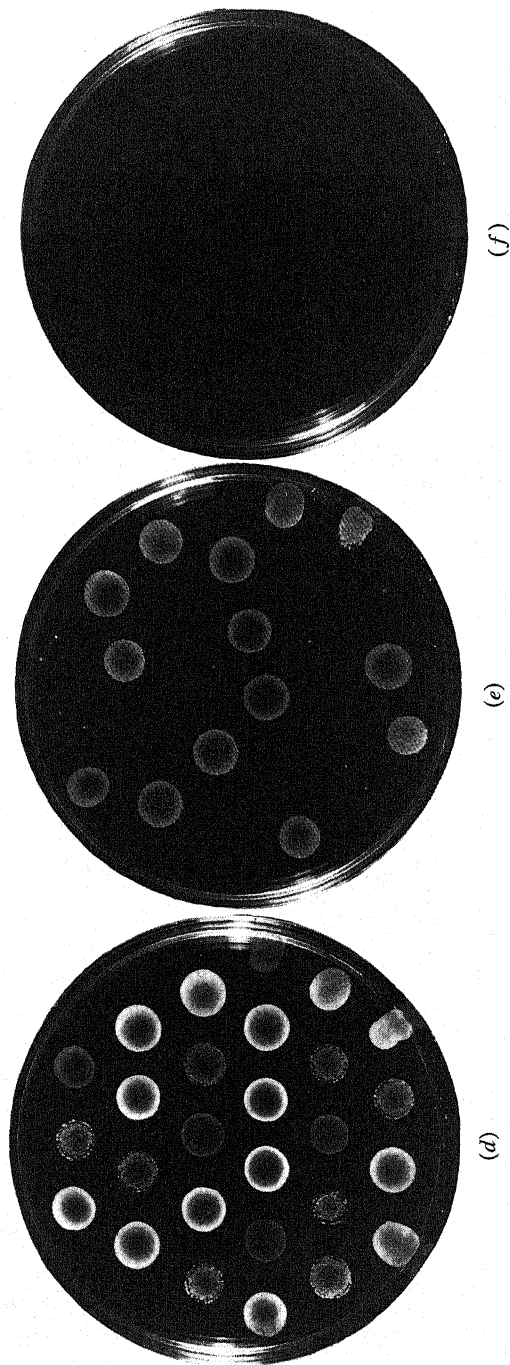
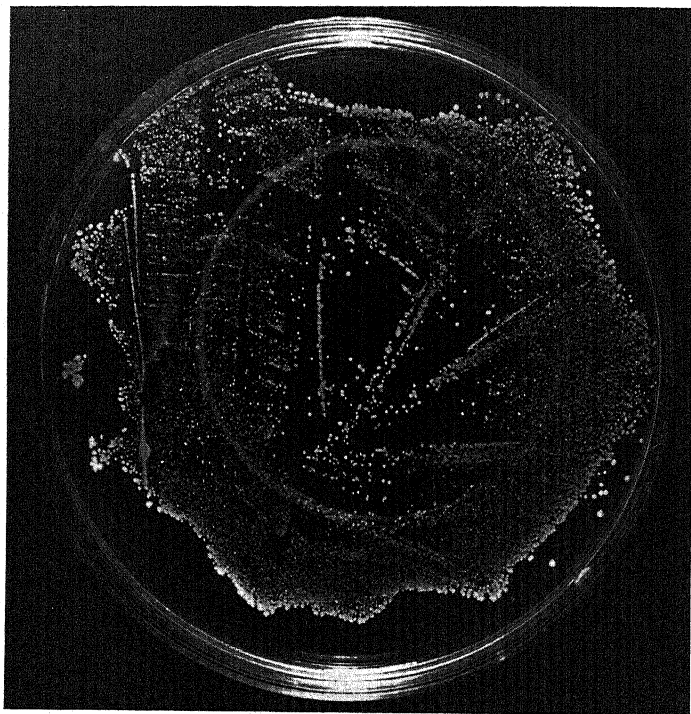
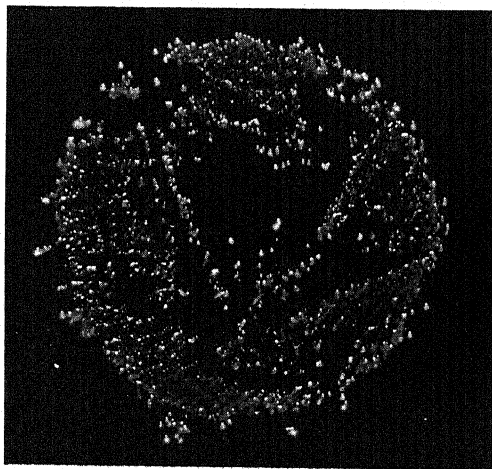


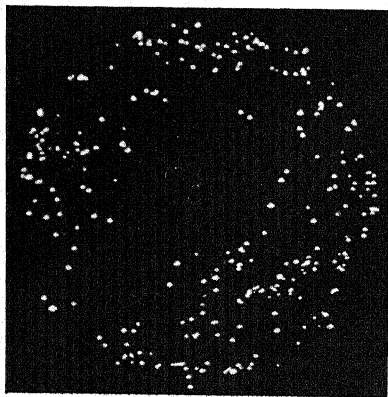
FIG. 1. Drug-containing agar inoculated with 27 strains of *Staph aureus* from clinical sources, (a) no drug (b) partially-purified ferrimycin, 10 $\mu\text{g/ml}$ (c) partially-purified ferrimycin, 1000 $\mu\text{g/ml}$ (d) quinacillin, 0.15 $\mu\text{g/ml}$ (e) quinacillin, 0.3 $\mu\text{g/ml}$ (f) quinacillin, 0.6 $\mu\text{g/ml}$.
The penicillin gives a relatively sharp end-point but ferrimycin-resistant colonies are apparent even on agar containing 1000 $\mu\text{g/ml}$ of the antibiotic.



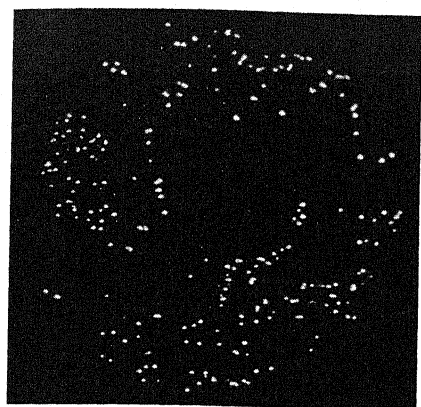
(a)



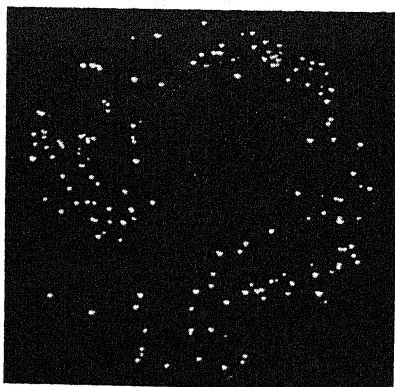
(b)



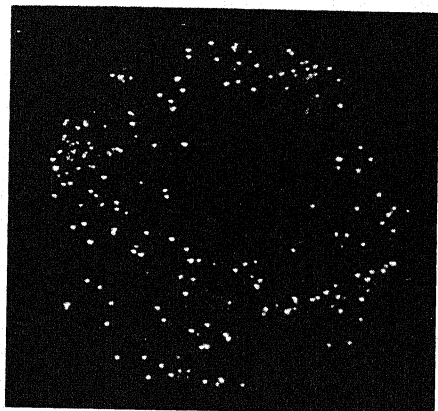
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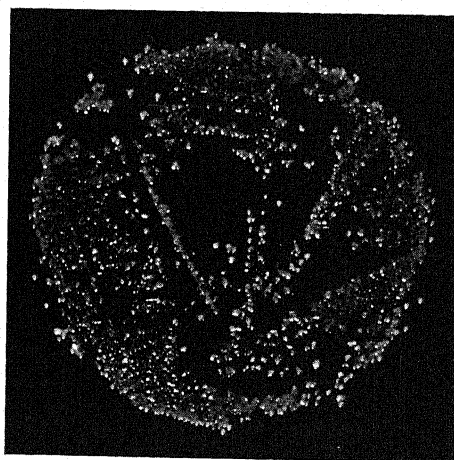
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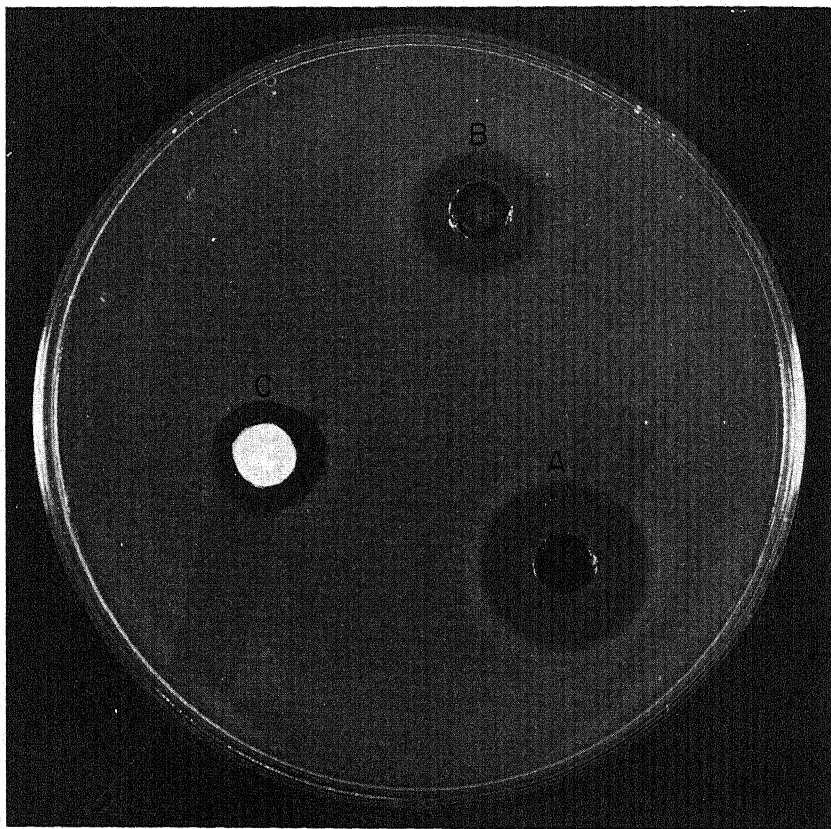
(g)

FIG. 2. Use of the Lederberg technique to isolate ferrimycin-resistant and sensitive *Staph aureus* cells. About 10^3 cells were spread on the parent drug-free plate (a); after incubation replicas were made on drug-free agar (b and g) and on agar containing 500 $\mu\text{g}/\text{ml}$ of partially-purified ferrimycin (c-f). Congruent colonies on replicas on drug-containing agar indicate the location of resistant clones of cells on the parent plate.

the Lederberg technique in attempts to obtain cultures of ferrimycin-resistant and ferrimycin-sensitive *Staphylococcus aureus* cells, which have not been in contact with the antibiotic, for biochemical studies (Fig. 2).

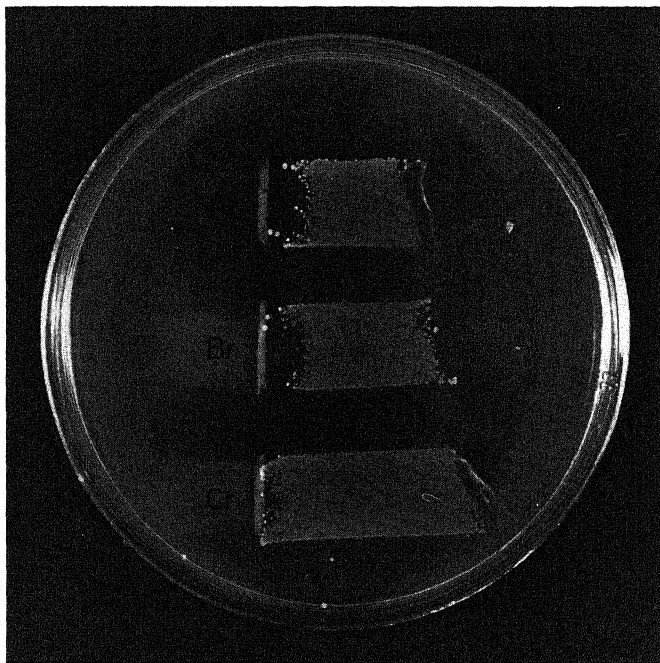
Bactericidal testing

Bactericidal testing consists of contacting a population of bacterial cells with an antibacterial agent under predetermined conditions, separating the treated bacteria from the antibacterial agents and then estimating the number of viable organisms on drug-free medium. The separation of treated cells

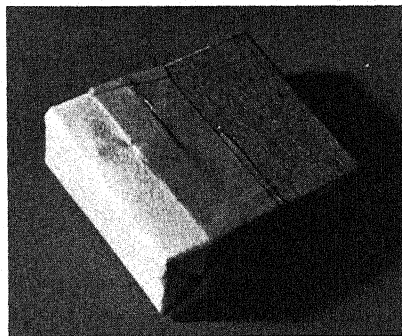


(a)

FIG. 3. The use of filter paper replicas for bactericidal testing. (a) Primary plate seeded with *Staph. aureus* showing zones of inhibition and areas from which replicas were taken, 0.5% hexachlorophane in 75% alcohol (A), in water (B), in cream formulation (C).



(b)



(c)

FIG. 3 (b) Replica plate showing zones of lethal action. The pencil lines indicate the edge of the cups. Ar, Br and Cr are the replicas from A, B and C respectively.

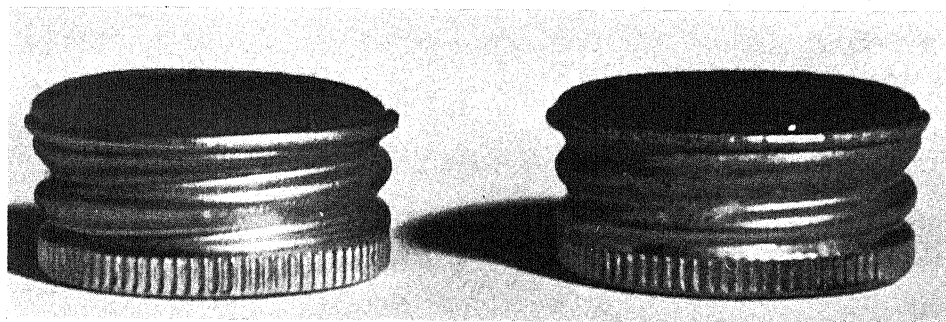
(c) Filter paper block for making replica.

from the antibacterial agent is most important and care must always be taken to ensure that "carry-over" of an inhibitory concentration of active material is not responsible for a false bactericidal effect. It is often useful to employ a single test to determine if a compound is bacteriostatic and bactericidal. The application of the Lederberg replica plating technique allows this and gives some indication of the number of surviving organisms without the necessity of time-consuming viable counts. We have used a modification of the method described by Elek and Hilson (1954) to determine the bacteriostatic and bactericidal activity of antibacterial agents both in solution and in formulation. Formulations or solutions are placed in cups cut out from seeded agar; alternatively filter paper discs may be used for liquids. After incubation replicas are made from the zones of inhibition on to agar plates, using moistened filter paper strips attached to wooden blocks (Fig. 3). Replicas are also made on to lightly seeded agar plates to determine if "carry-over" of active material has occurred.

A simple modification using agar to agar contact was devised in our laboratories (Parry, 1961). A replica is obtained on agar from the agar surface upon which a test organism and the bactericidal compound or formulation under investigation have been in contact. The replica agar is conveniently contained in suitable bottle-caps. A control replica is made to test for "carry-over" of bacteriostatic amounts of the test compound with a bottle-cap containing seeded or unseeded agar. If the latter is used it is later lightly streaked with the test organism. Bactericidal activity is indicated by absence of growth on the test replica and growth on the control seeded, or streaked, one (Fig. 4). The technique can be used to detect bactericidal activity during the routine screening of new compounds for bacteriostatic activity. Test organisms are inoculated on to the surface of nutrient agar, containing dilutions of test compounds, with a multipoint inoculator (Hale and Inkley, 1965). After overnight growth the bacteriostatic activity of the compounds is noted and then replicas are made from inoculated areas where growth has been inhibited. We use 12 mm aluminium test-tube caps ("Oxoid") to contain the agar for the replicas (Fig. 4). At the same time a "carry-over" control replica is made from an uninoculated area of the plate and the surface of this replica is inoculated with the relevant organism. Absence of growth from the first replica, but not from the second, indicates that the test compound has killed the cells during the overnight period of contact. Further tests can then be made using shorter periods of contact.

Skin and wounds

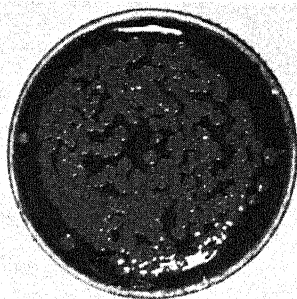
The replica method provides a relatively simple means of determining the number of bacteria on the skin surface and the effect on them of antiseptic



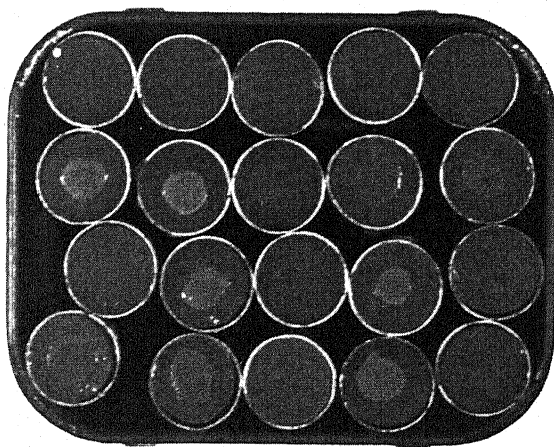
(a)



(b)



(c)



(d)

FIG. 4. Agar to agar replicas for bactericidal tests. (a) cap containing agar. (b), (c) replicas from inhibition zones on seeded agar; absence of growth in the centre (b) indicates bactericidal activity whereas growth over whole area (c) indicates absence of bactericidal activity. (d) replicas from agar-dilution tests (see Plate 1) on agar in test-tube caps.

treatments. Exact areas of skin can be examined more easily than with washing or swabbing methods and there is probably less danger of "carry-over" in antiseptic evaluation tests when a replica method is used. Direct impressions of organisms on the skin may be made with agar caps or discs (Lawrie and Jones, 1952). The dried agar surface is applied directly to the skin and then the bacteria are allowed to grow up *in situ*. In the evaluation of topical antibacterial preparations replicas are made before and after treatment, the regime of sampling the flora depending on the type of preparation under test. The incorporation of a drug inhibitor in the agar used for direct replicas is advisable. In any case care must always be taken to check for the "carry-over" of inhibitory concentrations of antiseptic by taking identical impressions and seeding the surface with a suitable suspension of bacteria. Velvet may also be used for the transfer of bacteria from the skin (Verdon, 1961) and has the advantage that there is rather less "carry-over" of antiseptic than with direct impressions. We prepare the velvet by glueing it to the cap of a "Universal container" so that the bottle may be used as a handle, or to tins that fit into Petri dishes. Such velvet contact areas can be used a number of times if steam sterilized before use but the velvet must be frequently examined and discarded if the pile has become matted. The velvet is first touched on to a plate of culture medium to pick up a thin layer of agar. The moistened surface is then applied to the skin and pressed back on to the surface of the agar plate. Further impressions may be made on to selective media or the first plate may be incubated and then replicas from it made on selective media.

Scotch tape has also been used as a contact area to enumerate the skin flora. Updegraff (1964) recommends Scotch brand 850 industrial tape since this does not possess bacteriostatic properties. A strip of this tape, which is sterile as manufactured, is pressed firmly on to the skin and then laid on a Petri dish, adhesive side up. Nutrient agar is poured over the tape and after incubation the organisms are enumerated.

The Lederberg technique has been used for the study of the bacterial flora of wounds (Gorrill and Penikett, 1957). An initial print from the infected area is taken with a sterile velvet pad and this is used to inoculate suitable selective or antibiotic-containing media. We have adapted this method to determine the bactericidal effect of a topical agent. An experimentally infected wound is produced by scarification of the shaved skin of a guinea pig followed by surface inoculation with a suitable bacterial suspension of *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Replicas are made with velvet before, and at intervals after, the topical application of a drug in solution or formulation. These are compared with those from wounds treated with the drug vehicle alone.

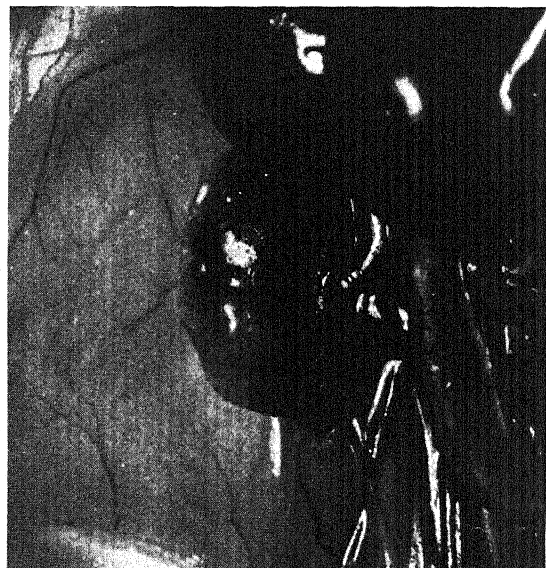
Isolation Techniques in the Evaluation of Chemotherapeutic Agents *In Vivo*

When new drugs are evaluated against experimental infection in laboratory animals, bacteriological isolation methods are frequently used to obtain pure cultures of the pathogen from the host. Sometimes, for instance, bacteria have to be passaged through experimental animals in order to enhance, or maintain, the virulence of the organisms for the experimental host and they then have to be repeatedly isolated from the animal body. In protection experiments with new drugs the infecting organisms are isolated from dying animals to ensure that the animals are succumbing to the administered pathogen and not to a naturally-acquired infection. When a resistance-lowering substance, such as gastric mucin, is employed to help initiate the experimental infection this can be particularly important as gastric mucin has been known to exacerbate a latent infection in laboratory mice (Engley, 1954). Similarly, attempts are made to recover infecting pathogens from treated animals to determine whether the bacteria have been completely eradicated by the drug or if resistant mutants have been selected *in vivo*.

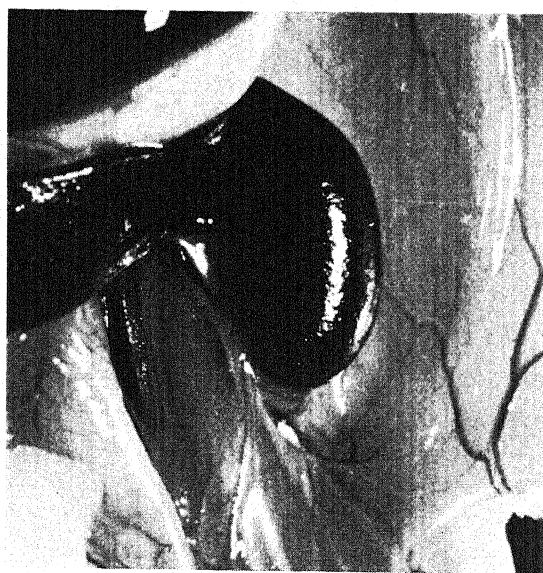
At times, too, the normal host flora of experimental animals or human volunteers has to be examined to determine if the administration of a new chemotherapeutic agent has altered the balance of organisms. Here, again, isolation techniques have their part to play.

Experimental infections leading to the death of laboratory animals are not always attainable or, indeed, desirable in view of their severity. In these cases it is sometimes possible to evaluate a new agent by assessing its ability to eradicate organisms from localized sites in the animal body and isolation techniques are employed to obtain the pathogen in pure culture for enumeration. The effect of chemotherapeutic agents on many types of experimental infections has been assessed in this way. A variety of isolation techniques has been used in these studies to evaluate drugs against mycobacteria (see Youmans and Youmans, 1964), staphylococci (McCune *et al.*, 1956) shigellae (Hoskins and Dack, 1946) and dermatophytes (O'Sullivan, 1961) to quote but a few examples.

We have recently been interested in the evaluation of potential drugs against *Candida* localized in the intestine or in the vagina. In the enteric method (Lindh, 1959) a suspension of *Candida albicans* is given in lieu of drinking water. The effect of the drug, which is given in the diet, is determined by comparing yeast counts on faecal pellets from treated and control mice. The faecal pellets are first emulsified in a streptomycin/penicillin solution. Dilutions of this suspension are then spread on the surface of "Oxoid" *Candida* agar and the black yeast colonies counted after incubation. A vaginal infection is obtained by injecting a *Candida albicans* suspension



(a)



(b)

FIG. 5. Chronic lesions in kidney of mouse infected intravenously with *P. mirabilis*
(a) Infected kidney. (b) Normal kidney for comparison.

intravaginally to spayed rats in a state of permanent oestrus (Scholer, 1960). The effect of local or systemic treatment is assessed by plating vaginal scrapings on the surface of a selective medium and comparing the number of yeasts from treated and control rats. We have used either "Oxoid" *Candida* medium or "Oxoid" malt extract agar made inhibitory to bacterial contaminants by the addition of 0.035% (w/v) potassium tellurite and 0.02% (w/v) neomycin sulphate. The latter medium is preferable because it is easier to prepare and gives a clearer agar. In addition potassium tellurite and neomycin are more effective as bacterial inhibitors than the streptomycin and penicillin in the *Candida* medium. At the level recommended the latter allow the growth of some enteric bacteria which can give rise to dark colonies similar to those of *Candida* sp.

The intravenous injection of several species of bacteria can lead to infections which become localized in the kidney (Fig. 5) and there is considerable interest in experimental pyelonephritis caused by Gram-negative bacteria (see Jackson, 1965). It is difficult to establish such a chronic infection in mice for drug evaluation but when it is established the bacteria can be isolated from the kidneys and counted. The infected kidney has first to be disintegrated to liberate the organisms and in an investigation of the Mickel disintegrator (Mickle, 1948) for this purpose we found that the recovery of organisms was very variable. It was later found that they were rapidly killed in the disintegrator when physiological saline was used as the suspending medium. However the loss of bacteria was minimized when dilute nutrient broth or peptone water was employed (Table 1).

TABLE 1. The survival of *Proteus mirabilis* in different suspending media during oscillation in the Mickel disintegrator

Minutes of oscillation	% Cells surviving*			
	0.85% Saline	Quarter-strength Ringer solution	Dilute† nutrient broth	1% peptone water
0	100	100	100	100
2	0	4.2	97	67
4	0	1.3	85	57
6	0	0	75	67
8	0	1.3	77	32.5
10	0	0	64	42

* About 2×10^4 organisms in 10 ml of each suspending medium were oscillated in "Mickle" vessels containing No. 12 Ballotini beads. At intervals 0.1 ml aliquots were spread over the surface of MacConkey agar plates. After overnight incubation colonies were counted and expressed as a percentage of the count at zero time.

† Equal volumes of nutrient broth and quarter-strength Ringer solution.

This observation emphasizes the well-known importance of employing the correct diluent when enumerating bacteria (see Jayne-Williams, 1963). It also illustrates the difficulties involved in the quantitative isolation of pathogens from infected tissues during drug evaluation studies.

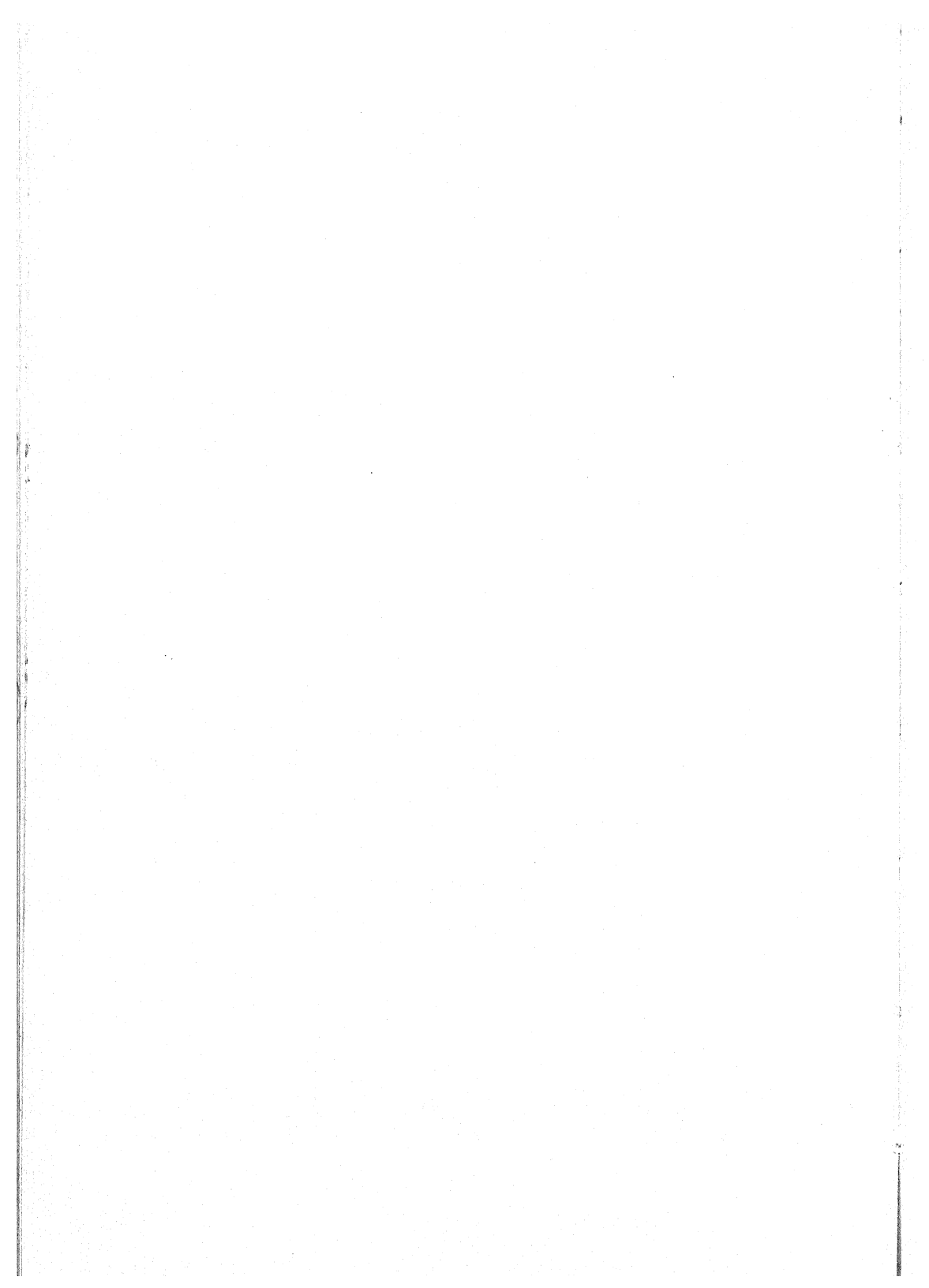
Acknowledgement

We thank Mr M. Spowage for help in the preparation of Figures 1 and 2 and Mr D. P. Adamson for the photography.

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The Isolation and Cultivation of Single Bacteria and their Spores by the Agar Gel Dissection Techniques

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The advantages of these methods are (1) they can be applied to a wide range of bacteria, both aerobic and strictly anaerobic, (2) the same batch of cleared agar can be stored and used for many types of organism, (3) since the isolates are transferred to the optimal culture medium at the end of the operations, the medium used may be fluid or solid, transparent or turbid, and (4) the apparatus for the actual isolations can be of simple construction, no micro-manipulator being necessary, but a microforge (De Fonbrune, 1949) is essential for efficient preparation of the microneedles.

Materials and Equipment

The agar gel

To remove dead bacteria which abound in agar, a 2.25% (w/v) solution of New Zealand agar in distilled water is clarified with "Hyflo Super Cel" diatomaceous earth (Koch-Light Laboratories) and is stored in sealed phials at pH 7.0-7.4. The concentration of agar is lowered by dilution to the optimum for micromanipulation before use, as determined by trial with the type of microinstrument to be used.

Casting of the agar blocks

The agar gel is cast in slabs between sterile microscope slides separated by glass strips 2.0 mm thick. The gel is cut into blocks 2.5×1.25 cm and 2.0 mm thick with a sterile stainless steel scalpel, each block being deposited on a sterile slide enclosed in a Petri dish with humidifying trough. Protection from desiccation is essential in order to maintain the plane upper surface of the block upon which manipulations are carried out.

Inoculation of the block

To avoid contamination of the sterile surface of the agar block by bacterial aerosols scattered during inoculation, the gel is protected by a sterile metal

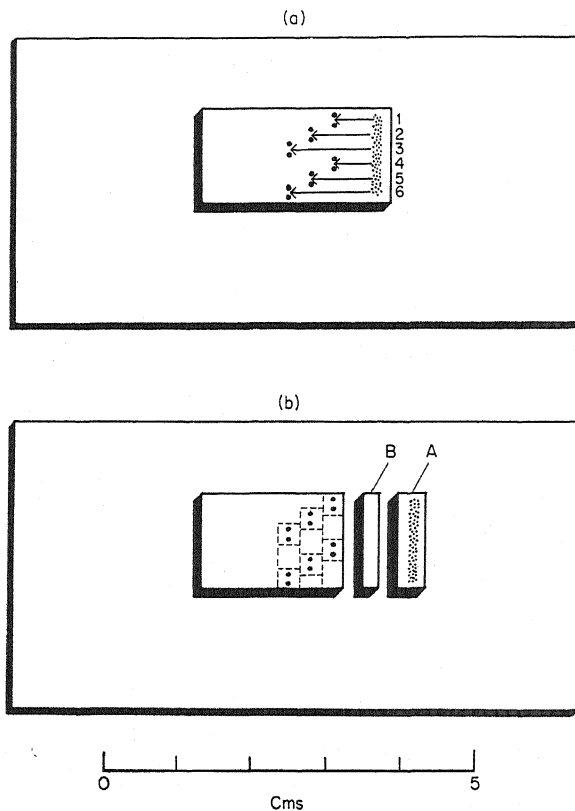


FIG. 1. (a) A block of agar gel mounted on a sterile slide, showing the routes taken by six successive isolates carried with a microneedle and the pairs of locating pits. The inoculated area is stippled. (b) The same block during dissection: A, the portion carrying the inoculated area; B, the adjacent section removed for sterility test. The broken lines indicate the knife cuts separating the portions of gel carrying the isolates.

shield except for a strip 2 mm wide at one end. A very light suspension of the required organism is streaked across the exposed portion of the upper surface with a wire loop 1 mm diameter and the fluid is rapidly absorbed by the gel, leaving the organisms on the surface (Fig. 1). After removal of the shield, the block is protected from aerial contamination and from desiccation by a Perspex cell supported by the surrounding slide, with access slots for the microinstruments and carrying a no. 0 coverglass 5.0 cm \times 2.5 cm, the lower surface of which is 0.9 mm above the gel surface.

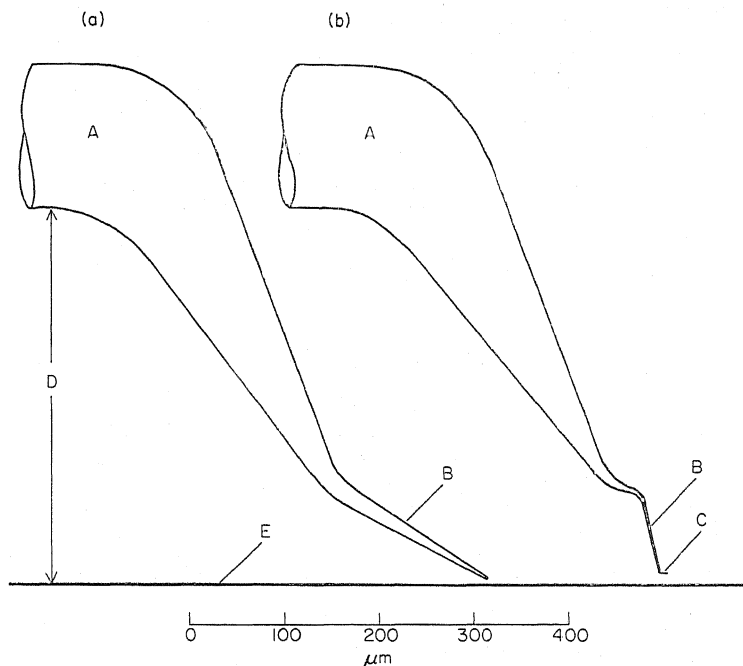


FIG. 2. (a) An angulated glass microneedle tip: A, the shaft; B, the tip. (b) A glass microloop: A, the shaft; B, the terminal filament; C, the loop, which lies in the plane of the gel surface. D, the clearance above the gel surface E.

Observation of the organisms

Phase-contrast microscopy is superior to all other methods, using a $\times 40$ objective of N.A. 0.65–0.70 and with a working distance of 1.0 mm below the coverglass, in conjunction with a long-working-distance phase-contrast condenser and a high-intensity illuminant with cooling trough and yellow-green filter. Condensation on the coverglass can be eliminated by a warm-air jet playing on its upper surface.

Alternatively, a catadioptric objective with long working distance allowing ample working space for the microneedle can be used, but at the expense of some loss in resolution and necessitating a great increase in illumination.

Method I: Carriage of the Isolate across the Gel Surface by Means of an Angulated Microneedle

The microneedle

This is formed from 1 mm soft glass rod drawn out over a minute coal-gas flame to approximately 0.15 mm diameter, this forming the shaft of the

needle 3.5 cm long and remaining attached to a 2.5 cm length of rod to serve as a handle. A simple microforge attachment to a microscope is necessary (Johnstone, 1953) to form the tip of the needle shaft, which is drawn out with a steep taper inclined downwards at 60° to the horizontal, to give clearance of approximately $400\text{ }\mu\text{m}$ between the shaft and the gel surface. The tip is then again drawn out at 30° inclination to the horizontal, terminating in a point $1.0\text{--}1.5\text{ }\mu\text{m}$ in diameter, depending upon the size of the organisms to be manipulated (Fig. 2).

A second microscope carries the microneedle, mounted horizontally in an attachment fitted to the nosepiece (Fig. 3). The focusing movements pro-

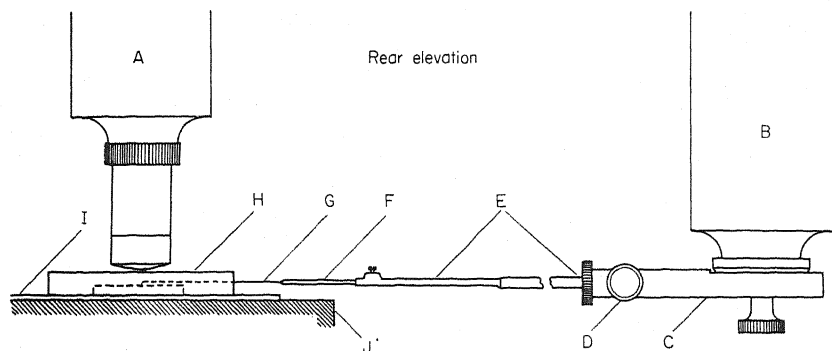


FIG. 3. The use of a second microscope as manipulator: A, body tube of observing microscope; B, body tube of second microscope; C, nosepiece attachment with clamping screw D for the microneedle holder E; F and G, handle and shaft of microneedle; H, sterile Perspex cell enclosing agar block and carrying the protecting coverglass; I, slide; J, mechanical stage. Coarse and fine adjustments must both move the body tubes.

vide coarse and fine motion for the needle tip in the vertical dimension; sliding of the microscope on the *smooth* bench surface enables the tip to be centred in the field of the observing microscope (Johnstone, 1969).

Micromanipulation of the organisms

The margin of the inoculum is first located with the $\times 10$ objective and the needle tip is guided into the Perspex cell, below the coverglass and above the surface of the agar block, being centred first to the $\times 10$ and then to the $\times 40$ objective. An organism at the margin of the inoculum is selected and the needle tip is gently lowered on to it. As the glass tip touches the gel, a minute pool of water exudes from the agar around it and the organism floats in the pool (Fig. 4).

By operation of the mechanical stage controls of the observing microscope, *the agar surface moves relative to the needle tip*, which latter remains centred in the field. Thus the isolate is carried across the sterile gel surface for 0.5–1.0 cm away from the inoculum and is readily identified as free from all other organisms of comparable size. On raising the tip, the isolate remains on the gel surface. If the organism escapes from the tip, it can be recovered by following the needle track backwards until it is found.

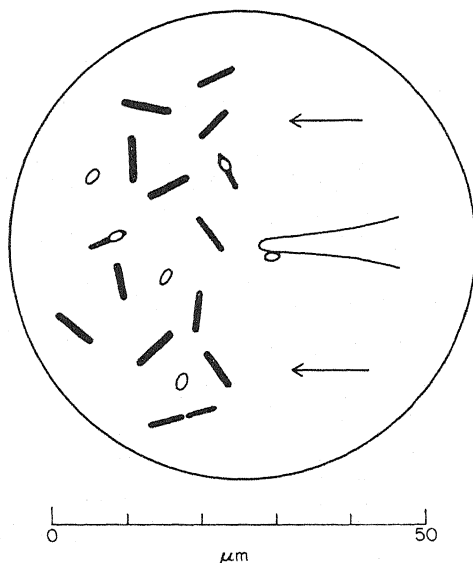


FIG. 4. Isolation of a single bacterial spore from a mixed culture using an angulated glass microneedle. The inoculated area appears on the left and the needle tip on the right, owing to image reversal. Arrows indicate direction of movement of the gel surface, imparted by the mechanical stage.

Location of the isolates

Each isolate is located by forming a pair of pits in the agar surface, one on each side of the organism and 1 mm apart. A marking needle is used having a V-shaped element of no. 40 s.w.g. platinum wire, terminating in a minute vertical tip at the apex of the V (Fig. 5). The platinum needle is mounted on the nosepiece of the second microscope in place of the glass microneedle holder and is centred, raised and lowered in the same way. By means of a 3-volt circuit, the platinum element can first be sterilized at red heat and then slightly warmed when in contact with the gel, to form a pit in its surface to one side of the isolate and then a second pit on the other side. The pairs of locating pits thus formed are visible to the unaided eye. Six isolations

can be made on the same block, being staggered to facilitate dissection of the gel (Johnstone, 1969).

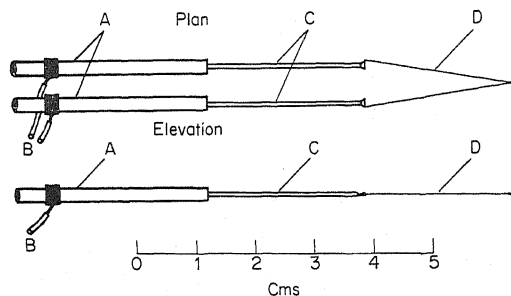


FIG. 5. The platinum marking needle: A, the electrically-insulated supporting steel rods; B, flexible cables; C, 24 s.w.g. platinum wires; D, the heating element of 40 s.w.g. platinum wire. The unit is mounted to be interchangeable with the microneedle holder in the nosepiece attachment.

Cultivation of the isolated organisms

Using a stereoscopic dissecting microscope, magnification $\times 5$, the slide and agar block are protected from aerial contamination by an enclosing cabinet and by a sterile plastic shield, with an access slot for the dissecting knives. The site of the inoculum is first separated and removed, using a sterile stainless knife and a platinum scoop (Fig. 1b). The portion of the gel between the inoculum and the nearest isolation site is then removed and must prove to be sterile if the isolations are to be accepted as valid.

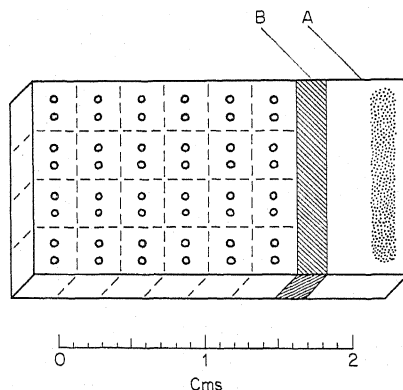


FIG. 6. A block of agar gel pre-marked in the agar punch for the deposition of 24 isolates with a microloop: A, the portion carrying the inoculum (stippled); B, the section tested for sterility. The broken lines indicate the knife cuts separating the portions of gel carrying the isolates.

The isolation sites are successively cut out of the agar block and are transferred with the sterile platinum scoop to separate tubes of the optimal medium for growth, either fluid or solid. If the latter, the small portion of agar is turned face downwards to trap the isolate between the gel and the surface of the medium.

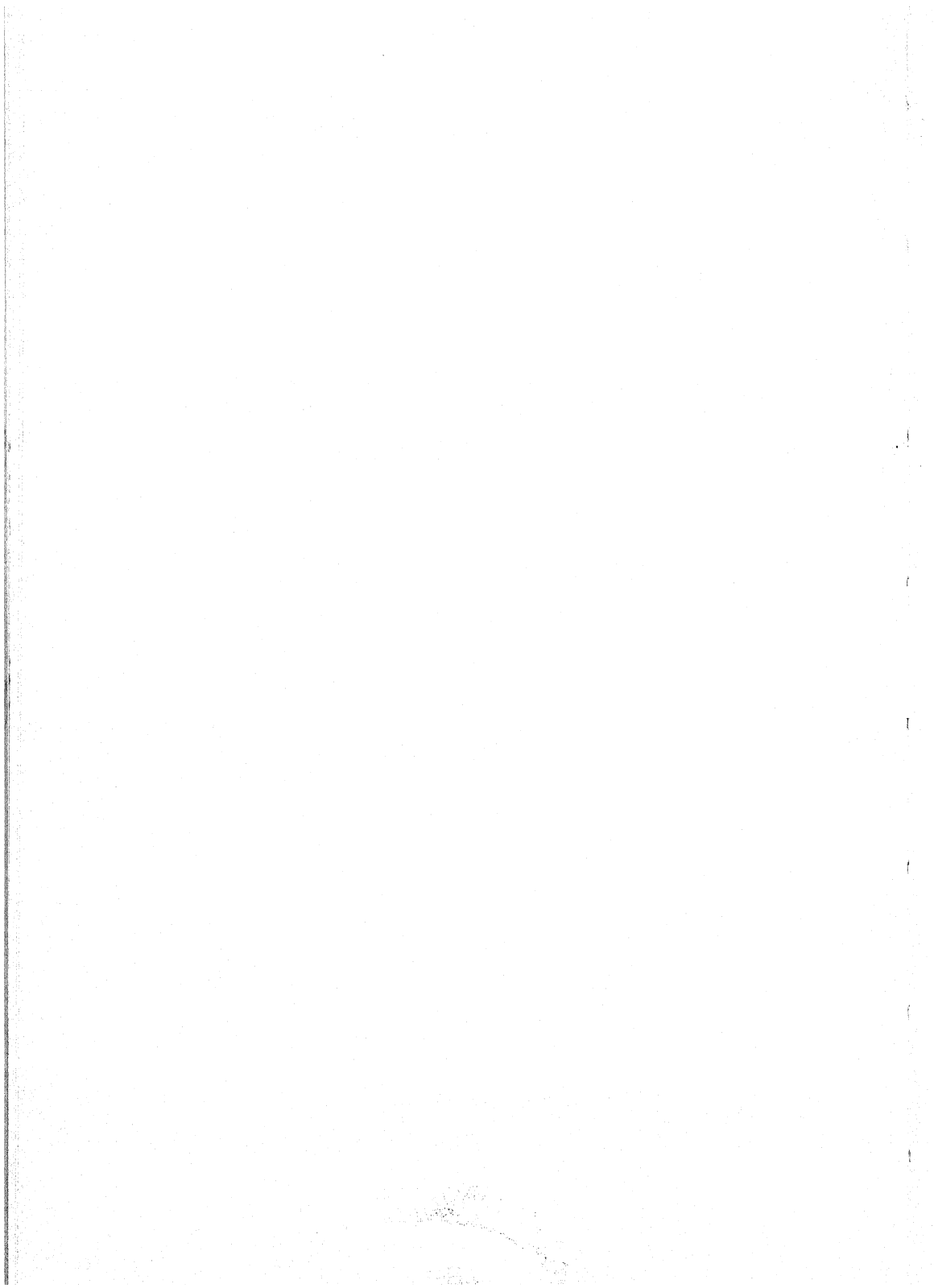
It is essential that the medium be of optimal composition, of correct reaction and oxidation-reduction potential if a high proportion of isolates are to prove viable. These requirements are much more stringent than for cultures receiving heavier inoculations.

Method II: Aerial Transfer of the Isolate to a Pre-marked Site by means of a Microloop

This has the advantage of great rapidity and is especially suitable for multiple isolations from one inoculum (Holdom and Johnstone, 1967). The agar block is pre-marked (Fig. 6) for 24 isolations (four rows of six sites at 3 mm intervals), in a sterile punch carrying 48 needle points. The isolation sites are arranged to register with the readings of the mechanical stage to facilitate rapid location. The microloop (Fig. 2) is formed in a high-power microforge at the end of a needle shaft and is of suitable size to pick up successively and to retain 6–12 selected organisms. The isolation sites are then in turn brought below the microloop, which is lowered to touch the gel between the pits. By means of a vibrator device, one or more organisms are readily ejected from the loop at the moment of contact with the gel. If more than one organism is seen to lie on the gel surface, the surplus organisms are again picked up and the operation is repeated at each successive site. Dissection of the completed block is carried out as described for Method 1 (Fig. 6).

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